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(71) Applicants *(for all designated States except US)*: INTROGENE B.V. [NL/NL]; Lange Kleiweg 151, NL-2288 GJ Rijswijk (NL). ACADEMISCH MEDISCH CENTRUM AMSTERDAM [NL/NL]; Meibergdreef 9, NL-1105 AZ Amsterdam (NL). HET NEDERLANDS KANKER INSTITUUT [NL/NL]; Plesmanlaan 121, NL-1066 CX Amsterdam (NL).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: OUDE ELFERINK, Ronald, Petrus, Johannes [NL/NL]; Dr. Koomansstraat 27, NL-1391 XA Abcoude (NL). PAULUSMA, Coenraad, Cornelis [NL/NL]; Daendelsstraat 25 bis, NL-3531 GB Utrecht (NL). BOSMA, Piter, Jabik [NL/NL]; Gildstraat 124, NL-3572 ES Utrecht (NL). BORST, Piet [NL/NL]; Meentweg 87, NL-1406 KE Bussem (NL). EVERS, Raymond [NL/NL]; Berlaarstraat 57, NL-1066 PJ Amsterdam (NL). KOOL, Marcel [NL/NL]; Van Walbeeckstraat 52-I, NL-1058 CV Amsterdam (NL).

(74) Agent: SMULDERS, Th., A., H., J.; Vereenigde Octrooibureaux, Nieuwe Parklaan 97, NL-2587 BN The Hague (NL).

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(54) Title: A FAMILY OF ORGANIC ANION TRANSPORTERS, NUCLEIC ACIDS ENCODING THEM, CELLS COMPRISING THEM AND METHODS FOR USING THEM

(57) Abstract

The present invention provides a novel family of organic anion transporters of which until now only one member was known. The family includes multispecific organic anion transporters related to the canalicular multispecific organic anion transporter. The cDNA encoding the latter is also provided. The rat and human cDNA are exemplified. Uses of nucleic acids based on this gene family and of cells comprising such nucleic acids as well as vectors comprising sequences thereof are also disclosed especially in the area of gene therapy.

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Title: A family of organic anion transporters; nucleic acids encoding them, cells comprising them and methods for using them

The present invention lies in the field of molecular biology and genetic engineering. It is particularly concerned with mechanisms of transport for substances across cell membranes. More in particular it is concerned with transport of cytotoxic substances from the inside to the outside of cells.

A group of proteins involved in transport of molecules across membranes is the group of so-called ABC-transporters (ABC: ATP-binding cassette). One member of this group called MRP1 (multidrug resistance-associated protein) has been identified as being involved in transporting organic anions across cell membranes. This protein thus transports different substances than the P-glycoprotein encoded by the MDR-1 gene (MDR: multidrug resistance). MDR-1 is involved in the occurrence of multidrug resistance of for instance tumor cells. Multidrug resistance is one of the major problems in chemotherapy of cancer. On the other hand providing cells with multidrug resistance may be very useful in rescuing for instance bone marrow when chemotherapy is applied. Thus on the one hand there is a need for being able to prevent transport of cytotoxic substances out of the cell while on the other hand there is a need to be able to enhance transport of cytotoxic substances from cells. The P-glycoprotein encoded by MDR-1 is not capable of transport of all cytotoxic substances; its binding specificity is limited to certain groups of molecules. MRP-1 has a different binding specificity in that it transports anionic organic compounds, possibly complexed or conjuged with other substances. In the liver a protein has been characterized functionally which is an ATP-dependent non-bile salt organic anion transporter called canalicular Multispecific Organic Anion Transporter. This protein has been thought to be identical to MRP-1.

The liver plays a major role in the detoxification of many endogenous and xenobiotic, lipophilic compounds. Detoxification is accomplished by transferase-mediated conjugation with glutathione-, glucuronide-, or sulphate-moieties, resulting in negatively charged, amphiphilic compounds which are efficiently secreted into bile or urine. Hepatobiliary excretion of these conjugates is mediated by an ATP-dependent transport system, the canalicular Multispecific Organic Anion Transporter (cMOAT), located in the apical (canalicular) membrane of the hepatocyte (1). The identification of a transport-deficient mutant rat strain, the TR$^-$ rat (2), has contributed to the functional characterization of cMOAT (1). These rats have an autosomal recessive defect in the hepatobiliary excretion of bilirubin glucuronides (3) and other multivalent organic anions including, glutathione-*S*-conjugates (e.g. leukotriene C_4), and 3-*OH*-glucuronidated and -sulphated bile salts (4). Thus far neither a protein nor a complete cDNA encoding cMOAT have been identified. Transport studies in plasma membrane vesicles from cells overexpressing the human Multidrug Resistance-associated Protein 1 (hMRP1) (5), demonstrated a role for hMRP1 in the ATP-dependent transport of the glutathione conjugates LTC_4 and dinitrophenyl glutathione (GS-DNP) (6). Because these substrates are also transported by the putative cMOAT protein, *MRP1* as stated before, is a possible candidate gene for cMOAT. A recent study suggested lateral as well as canalicular localization of the rmrp1 gene product in Wistar liver, but only a lateral localization in TR$^-$ liver (14) and suggested a role for MRP1 in the (defective) hepatobiliary excretion of organic anions in TR$^-$ rats. In our view, however, the extremely low MRP1 expression in liver (7,5) renders it unlikely that this gene product is responsible for biliary organic anion secretion. Furthermore, the transport defect in the TR$^-$ rat appears to be specific for liver (9), while MRP1 is expressed in all human tissues (7). We have now found that cMOAT is encoded

by a different gene and thus that a family of organic anion transporters exists.

The present invention now provides a nucleic acid comprising a sequence encoding at least a part of a member of a family of organic anion transporters, said nucleic acid comprising at least a gene family specific fragment of one of the sequences of fig.1a or fig.1b or figs 17, 18 or 19 or the complement thereof, or a sequence having at least 55%, preferably 70%, in particular 90% homology therewith. Of this family sofar only one member, mammalian MRP was known.

We hypothesized that cMOAT might be a liver-specific homologue of MRP1. To obtain a rat *mrp1* probe, we applied the polymerase chain reaction (PCR) on rat lung cDNA using nested degenerate oligonucleotide primers which were based on the highly conserved first ATP-binding cassette of the hMRP1 sequence (see experimental part). The 213 base pair product obtained shared 83% amino acid sequence identity with the corresponding region of the hMRP1 sequence. When analyzed on Northern (RNA) blot, this PCR fragment hybridized with a single, 9.5-kb, transcript in all Wistar and TR$^-$ rat tissues examined, with high expression in lung and testis, but no detectable expression in liver. Because this expression pattern resembled that of h*MRP1* in human tissues (7), we assumed that we had isolated a part of the rat homolog of h*MRP1*, rat *mrp1* (*rmrp1*). In order to find the putative *cmoat* gene, two rat liver cDNA libraries were screened, using the *rmrp1* fragment obtained as a probe (see the experimental part). This resulted in the isolation of a full length cDNA with a single open reading frame of 1541 amino acids (Fig. 1b). Based on similarity searches (10), the protein was identified as a new member of the ABC-transporter family (11), with modest identity to other members of the family. Highest overall identity was found with hMRP1 (47.6%) (5), yeast Cadmium Factor 1 (41.8%) (12), and the human Cystic Fibrosis Transmembrane Conductance Regulator (30.2%) (13). The amino acid sequence identity with hMRP1 ranged from 38-61% outside the ATP-binding

domains to 67% and 75% in the first and second ATP-binding domain, respectively. Recently, two different partial *rmpl* cDNA sequences were disclosed (14). One of these sequences comprised a 347 nt fragment that closely resembles the rat *cmoat* cDNA sequence found by us, said partial sequence is, however, not identical to *cmoat*. Moreover, no relation has been made between said partial sequence and the putative cMOAT protein. In contrast, it was suggested that a mutant *mrpl* gene is responsible for the cMOAT-deficient phenotype.

Northern (RNA) blot analysis of rat tissues with a 1-kb restriction fragment of our isolated cDNA, revealed three different transcripts, ranging from approximately 6.5 to 9.5-kb, with high expression only in liver, and low expression in kidney, duodenum, and ileum (Fig. 2A). These transcripts were strongly decreased (but not absent) in liver (Fig. 2B) and other tissues of the TR^- rat, which suggests that these transcripts were related to the defect in the TR^- rat. The three transcripts observed were probably derived from a single gene, because the level of all three transcripts was decreased in the TR^- rat. The decrease of this transcript in TR^- liver suggests that the isolated cDNA encoded cmoat.

To examine the expression level and the cellular localization of the cMOAT protein in hepatocytes, we produced a monoclonal antibody (mAb M_2 III-5) to a bacterial fusion protein containing the 202-amino acid carboxyl-terminal end of the sequence (see experimental part). On protein blots this antibody detected a protein of approximately 200-kD in the canalicular, but not the basolateral plasma membrane fraction of the Wistar rat liver (Fig. 3). This molecular weight was very similar to that of hMRP1 and in good agreement with the predicted molecular weight of the cMOAT protein. The 200-kD protein was completely absent from the canalicular membrane fraction of the TR^- rat (fig.3), which correlated with the decreased mRNA level in TR^- rat liver (Fig. 2B). Again, this finding was in good agreement with the defect observed in TR^- rats

which lack a functional transport system for organic anions in the canalicular membrane.

Thus we have isolated the complete cDNA encoding the cMOAT protein, which is deficient in the TR⁻ rat. Since the *cmoat* mRNA was not completely absent in TR⁻ liver it was possible to also amplify the complete TR- *cmoat* cDNA by PCR (see the experimental part) using various specific primer sets. To identify the nature of the genetic defect in TR⁻ rats, we sequenced the obtained cDNA. This revealed a 1-bp deletion at amino acid position 393, which results in a frame-shift and subsequent introduction of a stop-codon at position 401 (Fig. 4). This deletion results in the destruction of a *Nla*III restriction site which provided a means to quickly confirm the mutation in cDNAs from various tissues (see the experimental part). The very low mRNA expression in TR⁻ rats (Fig. 2B) might be due to the fact that the frame shift causes premature termination of translation and subsequent increased degradation of the mRNA.

Our results show a correlation between the *cmoat* gene, the absence of the gene product from the canalicular membrane, and the defined congenital transport defect in TR⁻ rats. In addition to the exclusive canalicular localization of cMOAT (Fig. 3), we have found that hMRP1 is routed only to the lateral domain of the plasma membrane of pig kidney epithelial cells (see experimental part). Our findings thus suggest a differential localization of MRP1 (basolateral) and cMOAT (canalicular) and imply that cMOAT and not MRP1 is involved in biliary organic anion transport. This contrasts to suggestions made in the literature (14). It was also suggested (14) that an isoform of MRP1 exists in rat liver which is derived from the same gene by alternative splicing based on the detection of two different sequences for the second ATP-binding domain and only one for the first ATP-binding domain. Our complete cDNA data, however, show that there are also two different sequences for the first ATP-binding domain in *mrp1* and *cmoat*. In fact, the two cDNA

sequences differ considerably throughout the entire molecule, thus indicating that MRP1 and cMOAT are encoded by two different genes.

We conclude that the MRP homolog, identified here, encodes the canalicular Multispecific Organic Anion Transporter, and that a 1-bp deletion, resulting in a truncated, non-viable, protein, is responsible for impaired transport of organic compounds from liver to bile in the TR^- rat. TR^- rats have the same phenotype as patients with the Dubin-Johnson syndrome, characterized by mild chronic conjugated hyperbilirubinemia (15). Isolation of the human homolog of *cmoat* is required to elucidate the nature of the defect in humans. Overexpression of hMRP1 confers resistance of human tumor cells to a number of cytostatic drugs (16, 17), and this resistance is dependent on intracellular glutathione levels (18). Apparently, both MRP1 and cmoat are involved in the excretion of organic anions from cells. Thus, overexpression of cMOAT, like that of MRP1, might also confer resistance to cancer cells against cytostatic drugs or their metabolites.

Using the rat *cmoat* gene we also found and isolated the cDNA encoding the human cMOAT protein. Now that it is known that these two exist other species of this family of transporters can be found using the present invention.

These transport mechanisms occur throughout the living world, so family members can be found in bacteria, bacilli, yeasts and fungi, plants, invertebrae, vertebrae, in particular in mammalians.

In addition to *MRP1* and *cMOAT* (*MRP2*), other *MRP* homologs encoding GS-X pumps are present in the human genome, considering that there are at least four *MRP* homologs expressed in *Caenorhabditis elegans* (56). We therefore searched the Expressed Sequence Tag (EST) library (57) for putative human *MRP* homologs, and found three more *MRP* homologs expressed in humans. We call these new *MRP* homologs *MRP3*, *MRP4*, and *MRP5*.

To investigate a possible role of MRP homologs in drug resistance, we examined a large set of (multi)drug resistant cell lines for the (over)expression of *cMOAT*, *MRP3*, *MRP4*, and *MRP5*. We find that especially *cMOAT* expression is elevated in several cell lines, selected for cisplatin resistance, and also in some sublines of the human non-small lung cancer cell line SW1573/S1, selected for doxorubicin resistance. The expression level of *cMOAT* correlates with the cisplatin but not the doxorubicin resistance of these cell lines. Although *MRP3* and *MRP5* were overexpressed in some resistant cell lines, no clear correlation between drug resistance and the expression levels of *MRP3*, *MRP4*, and *MRP5* has emerged from these studies as yet.

Preferred for the purposes of this invention are closely related members of the members identified by the sequences of fig.1a and fig.1b, most preferred those members which transport similar or the same compounds when expressed in a cell, or the closely related family members identified herein as MRP 3, 4 and/or 5. Most preferred is the human cmoat gene or its human family members and their products for their usefulness in for instance gene therapy and for their use in preparing blocking agents to the transporting product.

Further embodiments include but are not limited to a vector comprising a nucleic acid according to the invention and suitable means for replication, transduction and/or expression of said nucleic acid.

Preferably such a vector further comprises a gene encoding a therapeutically beneficial protein, which may be any protein having a beneficial effect under certain circumstances such as giving glutathion elevating activity, which enhances transport of anionic complexes or conjugates by the invented transporters.

Such vectors include vectors wherein the gene encodes at least a functional part of a gamma glutamyl cysteine synthetase or a UDP-glucuronosyltransferase.

Other vectors according to the invention include vectors wherein the therapeutically beneficial protein is another multidrugresistance related protein such as MDR1.

The invention further provides a cell comprising a nucleic acid or a vector according to the invention. Said cells may be any cells, preferred are bone marrow progenitor cells, in particular hematopoietic stem cells.

If said vector thus not encode additional desired functionalities apart from the cMOAT activity as disclosed above, said activity may be present on a separate vector to be introduced into said cell.

The invention also provides a method for providing cells with Canalicular Multispecific Organic Anion Transport protein activity, comprising transducing said cell with a nucleic acid or a vector according to the invention, as well as a method for enhancing Canalicular Multispecific Organic Anion Transport protein activity of cells according to the invention, comprising increasing the intracellular level of glutathion, glucuronide and/or sulphate. This may be done by contacting the cell with for instance glutathion esters, but also by providing additional genetic material as disclosed above. This may be done by cotransducing UDP-glucosedehydrogenase or sulphotransferase or any other means of enhancing such activity.

Thus the invention also encompasses methods for enhancing Canalicular Multispecific Organic Anion Transport protein activity of cells according to the invention, comprising enhancing the conjugating capacity and/or the complexing activity of said cell for sulphate, glutathion, glucuronide and the like.

On the other hand the invention provides a method for reducing Canalicular Multispecific Organic Anion Transport protein activity and/or the multidrug resistance of a cell comprising providing said cell with an antisense construct of a nucleic acid or a vector according to the invention, which antisense constructs are thus also part of the present invention. These methods can be used to block or at least

reduce transport of substances by the transporter protein according to the invention thus reducing resistance of for instance tumor cells to certain chemotherapeutic substances. Other ways of blocking the invented transporter are also part of the invention. These include methods of reducing the level of the conjugating or complexing molecules that enhance transport by the invented transporter. Antibodies to (in particular the extracellular domain of) the transporter.

For even further reducing multidrug resistance of for instance tumor cells said cells can be further provided with an antisense construct derived from another multidrug resistance related protein such as MDR1.

Proteins encoded by a nucleic acid according to the invention or obtainable by expression of a vector according to the invention are of course also part of the present invention, in particular proteins having Canalicular Multispecific Organic Anion Transport protein activity or Canalicular Multispecific Organic Anion Transport protein specific antigenicity comprising at least part of the sequence of fig.4 or being encoded by at least a part of the sequences of MRP 2, MRP 3 or MRP 4 (as given in the accompanying figures) or derivatives thereof having the same or similar function.

In the following a number of uses of the molecules, cells and methods of the invention are disclosed.

The invention enables the use of a nucleic acid according to the invention or a protein according to the invention in the diagnosis of Dubin-Johnson disease, Rotor disease or another disease involving Canalicular Multispecific Organic Anion Transport protein, as well as the use of a nucleic acid according to the invention or a protein according to the invention in the treatment of Dubin-Johnson disease, Rotor disease or another disease involving Canalicular Multispecific Organic Anion Transport protein.

Furthermore the nucleic acids according to the invention can be used as a selectable marker gene.

The members of the gene family disclosed herein have several useful applications in the context of gene therapy.

The concept of gene therapy has a very broad range of applications with one common denominator and that is the transfer of additional, new or corrected genetic information into cells which have a genetic or acquired defect. Examples of genetic disorders eligible for gene therapy are cystic fibrosis, Duchenne's Muscular Dystropy, cancer, Gaucher disease, Crigler Najjar and Dubin-Johnson syndrome. Examples of acquired diseases are cancer, viral and parasitic diseases. In addition, gene transfer can augment the efficacy of conventional therapies. Vehicles for the transfer of genes into target cells and tissues include vectors of viral and non-viral origin. Among the viral vectors murine based retroviruses and human based adenoviruses are the preferred embodiments.

Retroviruses are RNA viruses which efficiently integrate their genetic information into the genomic DNA of infected cells via a reverse-transcribed DNA intermediate as a proviral copy. Integration into the host's genome and the fact that parts of their genetic material can be replaced by foreign DNA sequences make retroviruses one of the more lucrative vectors for gene delivery in human gene therapy procedures, most notably for gene therapies which rely on gene transfer into dividing tissues. Recombinant murine retroviruses have been the vectors of choice since the start of gene therapy and several clinical trials using recombinant retroviruses are ongoing. In order to generate a recombinant retrovirus which carries the cDNA sequence of a particular gene one needs to introduce the retroviral construct into an appropriate packaging cell line. The retroviral construct carries the cDNA of interest and the cis acting elements for packaging and transcription of the viral RNA genome. The packaging cell line provides the trans acting factors needed for packaging: the *gag*, *pol* and *env* genes. Expression of the retroviral construct into the

packaging cell line results in the production of recombinant retroviral particles capable of transducing susceptible target cells and transferring a particular therapeutic gene. The recombinant retrovirus is stably integrated into the target cell genome and conferred to its daughter cells upon cell division.

Adenoviruses are non-enveloped DNA viruses. The genome consists of a linear, double stranded DNA molecule of about 36 kb. Recombinant adenovirus vectors have been generated for gene transfer purposes. Recombinant adenoviruses can be generated by co-transfection of two E1-deleted recombinant adenoviral DNA constructs, one of which comprising the sequences of interest, into an E1-expressing cell line. In contrast to retroviruses, adenoviruses do not integrate into the host cell genome, are able to infect non-dividing cells and are able to efficiently transfer recombinant genes in vivo. These features make adenoviruses attractive candidates for in vivo gene transfer into target cells which are difficult or impossible to treat ex vivo, such as cells of lung and liver.

Although the skilled artisan will be able to employ other vector systems than those exemplified here, such as Adeno Associated Virus (AAV), adenoviruses and retroviruses are preferred embodiments, because of the extensive experience with these viruses in gene therapy concepts.

Vectors comprising nucleic acids encoding and expressing functional members of the family of organic anion transporters disclosed in the present invention are of particular importance for the treatment of diseases caused by defects in these transporters. Examples of such diseases include Dubin-Johnson syndrome, Rotor syndrome and other cholestatic disorders. The human Dubin-Johnson syndrome

The earliest evidence for distinct canalicular transport systems for bile acids and non-bile acid organic anions came with the recognition of the human Dubin-Johnson

syndrome; this is a rare congenital chronic conjugated hyperbilirubinemia. The hepatic clearance of bilirubin and other cholephilic organic anions, like BSP and indocyanine green, is impaired in these patients, whereas bile acid clearance is normal. The urinary excretion of coproporphyrins, metabolic by-products of heme synthesis, is normal but the proportion of coproporphyrin isomer I is increased. The liver histology of this syndrome is characterized by lysosomal pigment accumulation.

Preferred target tissues for the genetic treatment of these diseases include the liver, gut and kidney.

Retroviral vectors comprising the nucleic acid sequences disclosed in this invention are constructed as exemplified in EP/95.201211.0 incorporated herein by reference. Recombinant retrovirus supernatant stocks are produced by introduction of the retroviral constructs in appropriate retroviral packaging cell lines. Adenoviral vectors comprising the nucleic acid sequences disclosed in this invention are constructed as exemplified in EP/95.202213.5 incorporated herein by reference. Adenovirus stocks are produced by transfecting the adenoviral construct into appropriate E1 complementing cell lines.

Hematopoietic stem cells (HSC) are the source for life-long production of all mature blood cell types. Therefore, genetic correction of HSC is expected to result in permanent mitigation of the clinical manifestation of inherited and aquired hematopoietic diseases. Of particular interest are Gaucher disease, thalassemia, sickle-cell anemia, AIDS, and others exemplified in WO93/07281. This makes HSC attractive targets for gene therapy. Currently, the established procedure for gene transfer into long-term repopulating HSC relies on the use of recombinant retroviral vectors. However, the gene transfer efficiency into human HSC is insufficient for the treatment of most hematopoietic diseases. This forms the bottle-neck for a broader application of bone marrow gene therapy. Therefore, it is

preferred to provide the recombinant retroviral vector with a marker sequence for positive selection of transduced cells. Selection for the presence of this sequence can be performed in vitro by culturing the transduced cells in the presence of a selective drug. Another approach is to select for transduced cells in vivo, following transplantation of transduced HSC. Both approaches can be taken by inclusion in the recombinant retroviral construct genes encoding transporter proteins conferring resistance to cytostatic drugs.

The members of the family of organic anion transporters disclosed in the present invention are important examples of genes that can be used for this purpose.

Another important embodiment of the present invention is the use of the disclosed members of the family of organic anion transporters to provide the hematopoietic system of cancer patients with resistance to chemotherapeutic drugs. This makes increased dose-intensity in the chemotherapeutic treatment of cancer possible. For most anticancer drugs increasing the dose-intensity results in increased response rates and a higher proportion of cures. Dose-intensity is the amount of drug administered per unit time, and can be augmented either by increasing the chemotherapy dose or by reducing intervals between cycles. Dose-intensive chemotherapy can produce complete regressions and improve survival in patients with historically refractory solid tumors and non-Hodgkin's lymphomas.Dose-response relationships have been demonstrated for many anticancer drugs. The major dose-limiting toxicity of many anticancer drugs is myelosuppression, which thus prevents optimum dose-intensity administration. Severe myelosuppression makes the patient particularly prone to opportunistic infections and is a frequent reason for curtailing chemotherapy before an adequate therapeutic response has been obtained. With a few exeptions (e.g., hormones) most anticancer drugs used in the clinic today, cause moderate to severe bone marrow toxicity

(e.g., vinblastine, epipodophyllotoxins, cisplatin, carboplatin, melphalan, methotrexate, alkylating agents, nitrosoureas, anthracyclines and anthraquinones).

Increasing the therapeutic index of myelosuppressive anticancer drugs by retrovirus-mediated transfer of genes encoding proteins conferring drug resistance is an attractive prospect. A recombinant retrovirus encoding a mutated dihydrofolate reductase (DHFR) that is highly resistant to the anticancer drug methotrexate has been constructed. Infection of murine bone marrow cells with this retroviral vector and subsequent reconstitution of lethally irradiated mice conferred protection from methotrexate-induced marrow toxicity. Furthermore, it has been demonstrated that transfer of the *MDR*1 cDNA into drug-sensitive cells can introduce drug resistance, in vitro as well as in vivo. Members of the family of drugs extruded from the cell by the *MDR*1 drug pump are e.g. anthracyclines, vinca alkaloids, podophyllotoxins, and colchicine. Etoposide, a commonly used podophyllotoxin of which the dose-limiting toxicity is restricted to the hematopoietic system, is also pumped by the *MDR*1 encoded drug pump albeit only poorly. The MDR related drugs have in common that they are lipophilic compounds derived from various natural products. In general, MDR cells are not cross-resistant to alkylating agents (e.g., chlorambucil and cyclophosphamide), antimetabolites (e.g., cytarabine, methotrexate, and 5-fluorouracil), cisplatin, carboplatin or melphalan. The members of the family of organic anion transporters disclosed in the present invention efficiently extrude organic anion compounds from the cell, including GS-DNP and chemotherapeutic agents such as the conjugated forms of cisplatin, carboplatin, etoposide, chlorambucil, and melphalan. This is shown in a nonlimiting example for cisplatin below. Therefore, introduction of the disclosed transporters into the hematopoietic system of cancer patients allows dose-intensification of frequently used chemotherapeutic drugs such as etoposide and are different

from those protected against by *MDR*1 or mutant DHFR. Therefore introduction of members of the disclosed invention into the hematopoietic system could lead to increased efficacy of cancer treatment.

The invention will be described in greater detail in the following experimental part.

Experimental

Example 1. Identification and isolation of the rat cmoat (mrp2).

A 213-bp PCR product was obtained from rat lung cDNA after first round amplification with degenerate primers corresponding to amino acid residues 678-648 (forward) and 770-776 (reverse), and subsequent second round amplification with nested primers corresponding to amino acid residues 694-700 (forward) and 760-766 (reverse) of the *hMRP1* sequence (5).

Partial cDNA clones were isolated from a rat hepatocyte cDNA library (23) which was screened with the 213-bp probe according to standard procedures (24). From a 4.5-kb positive clone a 5'-located, 0.6-kb *Hph*I restriction fragment was used to screen a gt10 5'-stretch rat liver cDNA library (Clontech, Palo Alto). A 0.8-kb overlapping clone was obtained from which a 0.6-kb *Ava*II probe was isolated to rescreen the same library, resulting in the isolation of another overlapping clone. The 5' end of the cDNA was obtained using the anchored PCR procedure [M.A. Frohman, M.K. Dush, G.R. Martin, Proc. Natl. Acad. Sci. U.S.A. 85, 8998 (1988)]. cDNA synthesis was carried out with 5 µg of total RNA isolated from Wistar rat liver and random hexamer primers using Superscript Reverse Transcriptase II. After purification the cDNAs were tailed with a synthetic oligonucleotide anchor sequence using a 5'-RACE kit (Life Technologies, Gaithersburg). Two rounds of nested PCR (96°C, 30sec; 60°C, 30sec; 72°C, 45sec) using an anchor specific primer and two *cmoat*-specific primers (5'-tgtccagtatcttctgtgagcg-3'(first round), 5'-aacacgacgaacacctgcttggc-3'(nested)) resulted in the isolation of the missing 5'-sequence. Probes were labeled with [a-^{32}P]dCTP using random primers. Hybridization of the filters was performed at 65°C in 0.5 M $NaPO_4$ (pH 7.0), 2 mM EDTA, and 7% SDS (hybridization solution), for 20 hours.

Filters were washed four times in 2x SSC, 1% SDS for 30 min at 65°C, and autoradiographed. Nucleotide sequences were determined by the dideoxy-nucleotide chain method [F.Sanger, S. Nicklen, A.R. Coulson, Proc. Natl. Acad. Sci. U.S.A. 74, 463 (1977)]. The *cmoat* sequence is being submitted to the Genbank database and is available under accession number L49379.

A fusion gene, consisting of the gene for the *Escherichia Coli* maltose-binding protein, and the 3' part of the *cmoat* cDNA corresponding to amino acid residues 1340-1541, was constructed in pMal-c [C.V. Maina *et al.*, *Gene* **74**, 365 (1977)]. The fusion protein was produced in E.coli strain JM101 and purified by amylose resin affinity chromatography. Mice were injected three times over six weeks with 200 µg of the purified protein. The first injection was in the presence of Freund's complete adjuvant, and the subsequent boosts in Freund's incomplete adjuvant. Two weeks after the final boost with a glutathione-*S*-transferase-cMOAT fusion protein, splenocytes were isolated and fused with myeloma cells. Hybridomas were screened by ELISA with the glutathione-*S*-transferase-cMOAT fusion protein and subsequently tested for positivity in Western blots.

cmoat cDNA was amplified from liver, kidney, ileum and duodenum from both Wistar and TR^- rats using primers corresponding to amino acid residues 366-375 (forward) and 451-458 (reverse) of the *cmoat* sequence. The obtained PCR product was digested with *Nla*III. In all PCR products from TR^- rat digestion produced two bands of 206 and 66 bp whereas in the Wistar three bands of 83, 122 and 67 bp were observed.

Total RNA was extracted using the acid-phenol single step method [P. Chomczynski and N. Sacchi, *Anal. Biochem.* **8**, 148 (1987)]. Poly(A)$^+$ RNA was isolated using the polyAtract mRNA system III (Promega, Madison). RNA was fractionated on a 0.8% denaturating agarose gel, transferred to Hybond N^+ nitrocellulose membrane filters and hybridized with a [a-

^{32}P]dCTP-labeled 213-bp rat lung *mrp*1 probe and a 1-kb *Hind*III/*Ava*II fragment of *cmoat* in hybridization solution (11) for 20 hours at 65°C. Filters were washed 4x30 min in 0.2x SSC/0.1% SDS at 65°C and autoradiographed. A ^{32}P-labeled 1.2-kb *Pst*I fragment of the rat glyceraldehyde-3-phosphate dehydrogenase cDNA [Ph. Forth *et al.*, *Nucleic Acid Res.* 13, 1431 (1985)] was used to estimate variations in RNA loading.

Canalicular and basolateral membranes were isolated as described by [P.J. Meier, E.S. Sztul, A. Reuben, J.L. Boyer, *J. Cell Biol.*, 98, 991 (1984)]. Membranes, containing 50 µg of protein were fractionated by 7.5% SDS polyacrylamide gel electrophoresis, electrophoretically transferred to nitrocellulose filters, blocked for at least 2h in PBS/M/T (phosphate-buffered saline containing 1% BSA and 1% milk powder and 0.05% Tween-20), and incubated with the monoclonal antibody (M_2 III-5 hybridoma culture medium diluted eightfold with PBS/M/T) for 2h. Immunoreactivity was visualized with peroxidase-conjugated rabbit anti-mouse immunoglobulins and subsequent staining with 3,3'-diaminobenzidine and 4-chloro-1-naphthol substrate. P-glycoproteins were detected using the monoclonal antibody C219 and peroxidase-conjugated rabbit anti-mouse IgG. Immune complexes were visualized by enhanced chemiluminescence detection.

Example 2. Isolation and characterization of the human cMOAT

The human homolog of the rat *cMOAT* cDNA was isolated using a 4 kb fragment of the rat *cMOAT* cDNA. The fragment was labelled as described for the rat *cMOAT* cDNA. The labelled probe was then used to screen a human lambda gt11 liver cDNA library. Three clones with inserts hybridizing with the rat cMOAT cDNA sequence were isolated and designated clone 12,7 and 20. Clone 12 contained an insert of 2716 nucleotides comprising coding sequence 130-2846. Clone 7 contained an insert of 2000 nucleotides comprising coding sequence 2517-3185. Clone 20 contained an insert of 2231 nucleotides comprising the coding sequence 3069-5300. Missing nucleotides 1-130 encompassing the translation initiation site were obtained from the WashU-Merck EST library, clone 1243479. Furthermore noncoding 3' sequences were found to be present in additional EST clones and were used to complete the full coding sequence of the human *cMOAT* cDNA. Clones 193244 and 199655 were used for this purpose and completed the full length sequence from 5300 to 5582 nucleotides.

Example 3. Transport experiments with rat cMOAT transfectants.

The rat *cMOAT* cDNA was cloned into the mammalian expression vector pSVK3 (Pharmacia). pSVK3-rat-cMOAT and pSVK3 with rat-cMOAT in the reverse orientation (pSVK3-rat-cMOAT/Rev) relative to the promotor were transfected into COS-7 cells grown in 75 cm^2 tissue culture flasks.Three days after transfection, the cells were used for GS-DNP transport experiments and analyzed for cMOAT protein expression using anti-cMOAT antibodies. For transport measurements the cells were washed with Hanks buffer and loaded with Hanks/^{14}C-CDNB at 15°C. Samples were taken after various time points. Input CDNB and cell mediated formation and transport of GS-DNP were separated by extraction of the samples with water-

saturated ethylacetate. The water phase which contains the excreted ^{14}C labelled GS-DNP was counted in a scintillation counter. Total protein was determined using the Lowry assay. GS-DNP efflux from the transfected cells was measured in a scintillation counter. Relative to COS-7 cells transfected with pSVK3-rat-cMOAT/Rev, cells transfected with pSVK3-rat-cMOAT excreted two fold more GS-DNP (See figure 5). This suggests that rat cMOAT transfected cells express a functional organic anion transporter protein in line with the expression of a protein reactive with anti-cMOAT antibodies.

COS-7 cells transfected as described above were also used to isolate membrane vesicles and perform transport experiments. For this purpose cell homogenates were prepared from transfected COS-7 cells and were centrifuged over a discontinous gradient of 19, 38 and 56 % sucrose. The 38-19 % interface was collected and revesiculated and total protein content was determined using the Lowry method.The vesicle suspensions were incubated wuth ^{3}H-GS-DNP at 37°C in the presence of an ATP regenerating system

After the indicated time points the vescile suspensions were filtered. Then the filters were washed with ice-cold stop buffer (250 mM sucrose, 20 mM HEPES/Tris pH = 7.4) and counted in a scintillation counter. In agreement with the cell transport experiments, vesicles isolated from cells expressing rat-cMOAT exhibited GS-DNP transport above the level of transport observed with vesicles isolated from pSVK3-rat-cMOAT/Rev transfected COS-7 cells. This transport was completely dependent on the presence of ATP characteristic for a member of the ABC transporter superfamily (figure 6).

Example 4: Transport experiments with human cMOAT transfectants

We have attempted to express the human cMOAT protein in several mammalian cell lines and in the yeast *Saccharomyces*

cerevisiae with various expression constructs carrying the human cMOAT cDNA. From 80 independant human cMOAT transfected and cloned human S1 cells only 2 clones expressed the human cMOAT protein at low levels. These expressing cells were tested for functional cMOAT by plating the cells in varying concentrations of doxorubicin, vincristin and cisplatinum.

The human LLC-PK1 cell line was also transfected with the same human cMOAT DNA construct and 90 clones were screened for expression. None of these clones expressed the human cMOAT protein as detected with antibody M_2III-6. In contrast, expression of human cMOAT in yeast was also studied and was high after the translational core sequences of the human cMOAT cDNA were converted to yeast consensus sequences.

However in both cases no active cMOAT mediated transport of GS-DNP could be observed. To investigate the role of cellular polarity in determining functional expression of the human cMOAT protein we have infected MDCK cells with an amphotropic retrovirus carrying the human cMOAT cDNA. For this purpose a HindIII-NcoI DNA fragment containing the complete predicted *human cMOAT* open reading frame was cloned into the retroviral vector pCMV-nec (Bender et al., 1987) resulting in a construct designated pCMV-neo-human-cMOAT. The retroviral amphotropic packaging cell line Phenix kindly provided by G.P. Nolan, Stanford University Medical Center, Stanford, USA) was cultured in Iscove's with 10% fetal calf serum. Phenix cells were transfected with pCMV-neo-human-cMOAT DNA using a commercially available calcium phosphate transfection kit (Gibco/BRL). After 16h at 5% CO_2, 37 °C, medium was changed and growth was continued for 48 h followed by collection of recombinant retrovirus containing medium. Storage of virus supernatants was at -20 °C. The MDCK cell line strain II (MDCKII; Louvard) was used for transduction experiments. For this purpose 2 x 10^5 MDCKII cells were seeded and incubated with a 5 ml 1/10 diluted virus stock in medium containing 30 mg Transfection

Reagent (DOTAP; Boehringer Mannheim, Germany). After 10 h medium was replaced with fresh medium. Thirty six hours after infection cells were trypsinized and seeded at dilutions varying between 1/12-1/64. Stably infected cells were selected for 2-3 weeks in medium with G418 at 200 mg/ml. Thirty clones were picked and analyzed for the presence of hcMOAT protein. Western blot analysis of crude membrane fractions of these clones revealed that several clones contained a substantial, but between individual clones variable, amount of human cMOAT. Two of these clones are shown in Figure 7. A weak signal was observed in wild-type MDCKII cells with a slightly higher molecular weight than hcMOAT after prolonged exposure. This might either represent canine cMOAT or another protein to which this mAb cross-reacts. The subcellular distribution of human cMOAT protein was determined in MDCKII-217 hcMOAT transfectants, which showed the strongest signal in Western blot analysis (See figure 7). Cells were grown to confluency on microporous polycarbonate membrane filters (3 mm pore size, 24.5 mm diameter, Transwell^{TM} 3414; Costar Corp., Cambridge, MA) at a density of 2 x 10^6 cells per well as previously described (8). For confocal laser scanning microscopy, cells were washed in PBS and fixed for 10 min in aceton at rt. Filters were incubated with mAb M_2-III-6 (undiluted) for 60 min. Antibody binding was detected with a FITC-labeled sheep anti-mouse IgG (1:50; Boehringer Mannheim, Germany). Filters were mounted with Vectashield (Vector Laboratories, Burlingame, CA) containing propidiumiodide (1 mg/ml) for counterstaining of nucleic acids. Cells were examined with a MRC-600 confocal microscope (Bio Rad, Hertfordshire, UK). Expression of hcMOAT protein was visualized by indirect immunolocalization using confocal laser scanning microscopy (CLSM). Clear staining was observed in approximately 50% of the cells (Figure 8 A), whereas in MDCKII wild-type only a very weak signal was detected (data not shown). Examination of the cells at the plane perpendicular to the membrane filter revealed that almost all human cMOAT immunostaining

was confined to the apical plasma membrane, although intracellular staining was observed in some cells (Figure 8 B).

To investigate whether human cMOAT expression in MDCK cells allows functional transport of GS-DNP the following experiment was performed. Export of [^{14}C]GS-DNP (GSH dinitrophenyl) from cells was determined by incubating cells with [^{14}C]CDNB ([^{14}C]-1-chloro-2,4-dinitrobenzene; 10 mCi/mmol) as described (8). The resulting hydrophilic GS-DNP only leaves the cell by active transport. Transport of GS-DNP across the apical and the basolateral membrane can be distinguished by growing cells as a monolayer on microporous membrane filters. Briefly, cells were grown on polycarbonate filters (see under immunocytochemistry) for 3-4 days. Two ml of medium (at room temperature) containing 2 mM [^{14}C]CDNB was applied to both the apical and basal compartment of the monolayer and 200 ml aliquots were taken at various time points. After extraction with 200 ml of ethylacetate radioactivity in 160 ml of the water phase was determined by liquid scintillation counting. The amount of radioactivity was corrected for the decrease in volume of culture medium. To determine intracellular radioactivity, cells were washed with cold PBS, filters were cut from the plate and counted directly in liquid scintillation fluid. The resulting pattern of GS-DNP export after exposing MDCKII, and the human *cMOAT* transfected clones is shown in Figure 9. MDCKII wild-type cells showed a substantial endogenous level of GS-DNP transport. Comparable transport to both the apical and basal compartment was measured in parental cells. Remarkably, apical GS-DNP export was substantially higher in both human cMOAT transfected clones, demonstrating that human cMOAT is active as a glutathione conjugate pump in these cells (figure 9). Comparing the Western blot data (Figure 7) with transport data suggests that there is a correlation between the amount of human cMOAT detectable and the level of apical GS-DNP transport. To exclude that differences in transport capacity between individual clones

were due to differences in GST activity, and therefore differences in conjugation capacity, the total amount of GS-DNP retrieved in cells plus medium after 20 min was calculated. These data revealed that all clones had comparable levels of GS-DNP formed (data not shown).

Example 5: A mutation in the human *cMOAT* gene causes the Dubin-Johnson syndrome.

The human Dubin-Johnson syndrome is an autosomal recessive liver disorder characterized by chronic conjugated hyperbilirubinemia. Patients have impaired hepatobiliary transport of non-bile salt organic anions a phenotype similar as has been described for the TR$^-$ rat, which has a defective cmoat. In view of the identical phenotypes of TR$^-$ rats and Dubin-Johnson patients, we have tested whether a mutation in the human *cMOAT* gene also underlies the transport defect in Dubin-Johnson syndrome. In this example we demonstrate that the human homologue of rat *cMOAT*, human *cMOAT* also subject of this invention, is deficient in a patient with Dubin-Johnson syndrome. Furthermore we show that we have used the DNA sequence of the human c*MOAT* cDNA to develop two diagnostic assays for Dubin Johnson syndrome.

We have studied a female,caucasian patient (age 54) who was diagnosed for Dubin-Johnson at the age of 20. She frequently complained of pains in the upper abdomen. General liver function was normal except for elevated conjugated (38 to 70 µM) and unconjugated (12 to 25 µM) serum bilirubin levels. It was not possible to visualize the gallbladder after administration of oral contrast reagent, a characteristic feature of Dubin-Johnson. The patient demonstrated a delayed plasma clearance of i.v. injected BSP, followed by a secondary rise in plasma BSP levels. At the age of 32 the patient underwent cholecystectomy. A characteristic black liver was observed and microscopic analysis of a liver section revealed mild fibrosis and the pigment accumulation indicative of Dubin-Johnson. Liver from

this patient was obtained by a needle biopsy. Normal control liver was obtained from surgical pathology specimens. Biopsies were fixed for histology in 4 % formaldehyde and embedded in paraffin. Skin fibroblasts from the patient and a normal control were obtained by skin biopsy and cultered in Ham F-10 (Life Technologies), supplemented with 10 % fetal bovine serum and antibiotics, at 37 °C.

Paraffin-embedded liver sections of Dubin-Johnson and control liver were examined for the presence and localization of the cMOAT protein, using monoclonal antibody M_2III-6. For this purpose formaldehyde-fixed paraffin-embedded liver sections were deparaffinized in xylene and rehydrated. Endogenous peroxidase activity was blocked with 0.3 % (v/v) H_2O_2 in methanol for 30 min. Before staining, the sections were pretreated with 0.01 M citric acid (pH 6.0) for 3 x 5 min at 100°C. The sections were blocked with normal rabbit serum for 10 min and incubated with monoclonal antibody M_2III-6 for 1 h. Immunoreactivity was visualized with biotinylated rabbit anti-mouse Fab_2 (Dako Copenhagen, Denmark), followed by streptavidin-conjugated horseradish peroxidase (Dako) in PBS/1% BSA, and subsequent staining with 3,3'-diaminobenzidine tetrahydrochloride and 0.02 % (v/v) H_2O_2 in PBS. P-glycoproteins were detected with monoclonal antibody JSB-1. All sections were (counter)stained with hematoxylin and mounted. The M_2III-6 antibody was produced against a bacterial fusion protein containing the 202-amino acid COOH-terminus of rat cMOAT; it cross-reacts with human cMOAT, but not with human MRP1. In human control liver, like in rat control liver, the antibody stained the canalicular membrane of the hepatocyte. In Dubin-Johnson liver , as in TR$^-$ rat liver, no canalicular staining was observed, indicating that this patient lacks the cMOAT protein (figure 11). A positive canalicular staining was observed in both Dubin Johnson syndrome and control liver with JSB-1, an antibody against P-glycoprotein, used as a positive control. To investigate the nature of the genetic defect, total RNA was isolated from

cultured fibroblasts obtained from a skin biopsy of both the patient and a normal control according to the acid-phenol single step method. cDNA synthesis was carried out with 6 µg of total RNA and random hexamer primers with Moloney Murine Leukaemia Virus Reverse Transcriptase (M-MLV-RT, Life Technologies, Gaithersburg, MD), at 37°C for 1 h, followed by 10 min 65°C to inactivate the M-MLV-RT. The complete *cMOAT* cDNA was amplified by the "touch down" PCR protocol from both patient and control fibroblast cDNA using five sets of *cMOAT*-specific primers:
5'-TAGAAGAGTCTTCGTTCCAGACGCAG-3'(forwardI) and
5'-GCAATTTCAGCAGCTGAGGACTCAC-3'(reverseI),
5'-AAATCCTGGTTGATGAAGGCTCTG-3'(forwardII) and
5'-TCCAGGTTCACATCTCGGACTCTGGC-3'(reverseII),
5-ACATCTGCCATTCGAGATGACTGC-3'(forwardIII) and
5'-CAACTCTCATGTCCCTCTGAGATGC-3'(reverseIII),
5'-TGAAGTTCTCCATCTACCTGGAGTACC-3'(forwardIV) and
5'-GATGATGGTCAGCTTCTCTCGGAGG-3'(reverseIV), and
5'-GTCATCCCTCACAAACTGCCTCTTCAGAATCTTAG-3'(forwardV) and
5'-CTGCTAGAATTTTGTGCTGTTCACATTC-3'(reverseV). PCR reactions were carried out in a Perkin Elmer GeneAmp PCR system 2400, in 1x *Taq* polymerase buffer (Life Technologies), 1.5 mM of $MgCl_2$, 0.5 mM of dNTPs, 400 nM of each primer, and 0.5 units of *Taq* polymerase. The PCR products were obtained after application of the "touch down" PCR protocol; the reactions were denatured at 96°C for 5 min, followed by five times 2 cycles with annealing temperatures of 72, 70, 68, 66, and 65°C respectively, and subsequent 30 cycles with an annealing temperature of 64°C. Each cycle started with 20 s at 94°C, 30s at the indicated annealing temperature, and 90s at 72°C. The PCR reaction was terminated after an extension step at 72°C for 10 min. PCR fragments obtained from fibroblasts were excised from agarose gel, purified, ligated into the TA-cloning plasmid pCR™II (Invitrogen, Leek, The Netherlands), and transformed into INVaF' competent cells (Invitrogen). White colonies were picked, grown overnight, and plasmid DNA was isolated using the alkaline lysis

method. Nucleotide sequences of 5-8 pooled clones were determined by the dideoxynucleotide chain method. Sequence analysis of multiple independent clones revealed a mutation in the patient at codon 1066 (CGA to TGA; arginine to stop-codon) (figure 12), which leads to premature termination of cMOAT protein synthesis, the normal protein being 1545 amino acids long (see also figure 10). The mutation results in the loss of a *TaqI* restriction site, and we have confirmed the absence of this site in the patient by *TaqI* digestion of her *cMOAT* cDNA (figure 13). From this observation we conclude that this patient is either homozygous for the mutation in codon 1066, or that the second allele does not give rise to a mRNA.

In conclusion, the identification of a mutation in human *cMOAT* in a patient with the DJS confirms the hypothesis that the TR^- rat is an animal model for Dubin Johnson syndrome and provides additional evidence that the *cMOAT* gene encodes the major transporter for organic anions in the liver canalicular membrane.Our demonstration of a low, but detectable expression of the *cMOAT* gene in fibroblasts in addition to a nucleic acid based diagnostic assay for Dubin-Johnson syndrome, allows a simple identification of this inherited disorder, without the need for liver biopsy.

Example 6: Identification and isolation of other members of the family of anorganic anion transporters.

6.1 Materials and Methods

6.1.1 Cell lines

All cell lines used in this study have been described in the literature before: the drug-sensitive and doxorubicin-selected MDR sublines of the non-small-cell lung cancer cell lines SW1573/S1 and COR-L23 (58-61); the small cell lung cancer cell line GLC_4 (62); the lung adeno carcinoma cell line MOR/P (61); and the leukemia cancer cell line HL60

(63); the T24 bladder carcinoma cell line and three CDDP-resistant sublines (45); the 2008 ovarium carcinoma cell line, two CDDP-resistant sublines and a Cd^{2+}-resistant subline (40, 41); the A2780 ovarium carcinoma and the HCT8 colon carcinoma cell lines and CDDP-resistant sublines of both (39, 47); the PXN94 ovarium carcinoma and the tetraplatin-resistant subline PXN94tetR (42); the GCT27 testicular carcinoma cell line and the CDDP-resistant subline GCT27cisR (44); the KB-3-1 epidermoid carcinoma cell line and a CDDP-resistant subline KCP-4 (37, 38). All cells were grown in DMEM or RPMI medium (Gibco, BRL), supplemented with 10% fetal calf serum, 2 mM glutamine, penicillin (50 units/ml) and streptomycin (50 µg/ml). All cells were free of *Mycoplasma* as tested by the use of the Gene-Probe rapid *Mycoplasma* detection system (Gen-Probe, San Diego, USA).

6.1.2 Clonogenic survival assays

The drug sensitivity of cells was determined in clonogenic survival assays in the continuous presence of drugs. Five hundred cells per well were seeded in 24-well plates and incubated for 24 hrs at 37°C. Drugs, of which concentrations were varied in 2-fold steps, were added and cells were incubated for 5-6 days at 37°C. After this the cells were stained with 0.2% crystal violet in 3.7% glutaraldehyde and colonies containing more then 50 cells were counted. The relative resistance was calculated as the ratio of IC_{50} (Inhibitory Concentration where 50% of the cells survives) of the resistant cell line to the IC_{50} of the parental cell line.

6.1.3 Cloning and sequencing of *MRP3*, *MRP4*, and *MRP5* cDNA

For the isolation of *MRP3*, *MRP4*, and *MRP5* cDNA, human cDNA clones were obtained from the I.M.A.G.E. consortium (64). Additional *MRP3* cDNA clones were isolated by screening a human liver 5' stretch plus cDNA library, oligo(dT) and random primed (Clontech, Palo Alto, USA), using a 1 kb

*Eco*RI-*Sac*I fragment of a human cDNA clone (no. 84966, Stratagene liver cDNA library #937224) as probe. Several overlapping cDNAs were isolated and sequenced. For *MRP4* the insert of a human cDNA clone (no. 38089, Soares infant brain 1NIB cDNA library) was sequenced, containing the 3'-terminal end of the gene. *MRP5* cDNA clones were isolated by screening a fetal brain cDNA library (Clontech, Palo Alto, USA), using the insert of human cDNA clone (no. 50857, Soares infant brain 1NIB cDNA library) as probe (J. Wijnholds, C. Mol, and P. B., unpublished results). Several overlapping cDNAs were isolated and sequenced. For sequencing the ABI 377 Automatic Sequencer was used. Sequence analysis was done using the GCG package of the Wisconsin University (20). All the sequences have been deposited with GenBank (*MRP3* accession number U83659; *MRP4* accession number U83660; *MRP5* accession number U83661).

6.1.4 RNA

Cytoplasmic RNA from cell lines was isolated by a Nonidet P-40 lysis procedure (24). Total cellular RNA from tissue samples obtained during surgery or at autopsy was isolated by acid guanidium isothiocyanate-phenol-chloroform extraction (65).

6.1.5 RNase protections

By PCR amplification of human *cMOAT* cDNA a 241 bp fragment corresponding to nucleotides (nts) 4136-4376 (49; GenBank accession number U49248) was generated. The primers used for amplification were 5'-CTGCCTCTTCAGAATCTTAG-3' (forward primer) and 5'-CCCAAGTTGCAGGCTGGCC-3' (reverse primer). For *MRP3*, *MRP4*, and *MRP5* RNA detection the following fragments were generated by PCR amplification: (i) for *MRP3* a 262 bp fragment was generated using the primers 5'-GATACGCTCGCCACAGTCC-3' (forward primer) and 5'-CAGTTGGCCGTGATGTGGCTG-3' (reverse primer); (ii) for *MRP4* a 239 bp fragment was generated using the primers 5'-CCATTGAAGATCTTCCTGG-3' (forward primer) and 5'-

GGTGTTCAATCTGTGTGC-3' (reverse primer); (iii) for *MRP5* a 381 bp fragment was generated using the primers 5'-GCATAACTTCTCAGTGGG-3' (forward primer) and 5'-GGAATGGCAATGCTCTAAAG-3' (reverse primer). All the fragments were cloned into pGEM-T (Promega, Madison, USA), resulting in the plasmids hcMOAT-241, MRP3-262, MRP4-239, and MRP5-381, and the sequences were confirmed. For RNase protections, a-^{32}p-labeled RNA transcripts were transcribed from *Not*I-linearized DNA of hcMOAT-241 and MRP3-262, using T7 RNA polymerase, or from *Nco*I-linearized DNA from MRP4-239 and MRP5-381, using Sp6 RNA polymerase. For *MDR1* RNA detection, a 301 bp *MDR1* cDNA fragment was used (nt positions 3500-3801 (66)), and for *MRP1* RNA detection a 244 bp *MRP1* cDNA fragment was used (nt positions 239-483 (7)). RNase protections were carried out according to Zinn *et al.* (67), modified by Baas *et al.* (58). Protected probes were visualized by electrophoresis through a denaturing 6% acrylamide gel, followed by autoradiography. In all experiments a probe for actin (68) was included as control for RNA input. The amount of *MDR1*, *MRP1*, *cMOAT*, *MRP3*, *MRP4*, or *MRP5* RNA relative to the amount of actin was calculated using a phosphorimager (Fuji BAS 2000, TINA 2.08b).

6.1.6 Protein analysis

Total cell lysates were made by lysing harvested cells in 10 mM KCl/1.5 mM $MgCl_2$/10mM Tris-HCl, pH 7.4/0.5% (wt/vol) SDS supplemented with 1 mM phenylmethylsulfonyl fluoride, leupeptin (2 µg/ml), pepstatin (1 µg/ml), and aprotinin (2 µg/ml). DNA was sheared by sonication and samples containing 40 µg of protein were fractionated by SDS/7.5% PAGE and then transferred onto a nitrocellulose filter by electroblotting. After blotting the filters were blocked for at least 2 hours in Blotto (Phosphate-buffered saline containing 1% bovine serum albumin, 1% milk powder, and 0.05% Tween-20), followed by incubation for 2 hours with the primary antibody in Blotto. cMOAT protein was detected with mouse monoclonal antibodies M_2III-5 or M_2III-6, generated against a bacterial

fusion protein containing the 202 amino acid COOH-terminus of rat cmoat (48). Immunoreactivity was visualized with peroxidase-conjugated rabbit anti-mouse immunoglobulins (Dako, Denmark) followed by enhanced chemiluminescence detection (Amersham, U.K.).

6.1.7 Fusion proteins of cMOAT, MRP3, and MRP5.
To test the cross-reactivity of the cmoat monoclonal antibodies with human cMOAT and other MRP homologs, fusion proteins were made of the *Escherichia coli* maltose-binding protein with COOH-terminal ends of human cMOAT, MRP3, and MRP5, respectively, using the plasmid vector pMal-c (69). The expression plasmids encoded, respectively, for cMOAT the 202-amino acid COOH-terminal end, for MRP3 the 190-amino acid COOH-terminal end, and for MRP5 the 169-amino acid COOH-terminal end. The fusion proteins were produced in *E.coli* DH5a and purified by amylose resin affinity chromatography (69).

6.1.8 Glutathione assay
Cells (1-2 x10^6 per well) were plated in triplicate in 6 wells plates in medium with or without drugs. 48 hrs after plating the cells were washed with phosphate-buffered saline and scraped in 10% perchloric acid. Precipitated protein was removed by centrifugation and the supernatant was neutralized by adding 0.5 M MOPS/5 M KOH. The concentration of total glutathione (GSH and glutathione disulfide (GSSG)) was determined according to the recycling method of Tietze (70).

6.1.9 Chromosome localizations
For the chromosome localization of *MRP3*, *MRP4*, and *MRP5*, radiation hybrid mapping was performed with *MRP3*, *MRP4*, and *MRP5* specific primers and two different cell panels, Stanford G3 (StG3; 71) and Genebridge 4RH (Gb4RH; 72). The primers used for amplification were: (i) for *MRP3* 5'-CTCAATGTGGCAGACATCGG-3' and 5'-GGGAGCTCACAAACGTGTGC-3';

(ii) for *MRP4* 5'-CCATTGAAGATCTTCCTGG-3' and 5'-GGTGTTCAATCTGTGTGC-3'; (iii) for *MRP5* 5'-CCTGTTTGGGAAGGAATATGA-3' and 5'-GGGTCGTCCAGGATGTAGAT-3'. For the PCR reactions 25 ng DNA, 2 ng/µl of each specific primer, 0.8 units Goldstar polymerase (Eurogentec, Seraing, Belgium) (*MRP3* and *MRP4*) or 1.5 units Amplitaq Gold polymerase (*MRP5*) were used in a total volume of 25 µl with 1.5 mM $MgCl_2$ and 100 µM of each dNTP at final concentrations. The PCR conditions were: initial denaturation 5 min 94°C (*MRP3* and *MRP4*) or 12 min 95°C (*MRP5*), followed by 42 cycles of 15 sec 94°C, 30 sec 58°C, and 45 sec 72°C. Final extension was for 10 min at 72°C. PCR products were resolved by 1 percent agarose gel electrophoresis and the cell line scored positive, negative or ambiguous for presence of the gene. Datafiles were submitted to the Stanford Human Genome Center or Whitehead Institute radiation hybrid mapping databases for placing of the *MRP* genes in context of the respective radiation hybrid map framework markers.

6.1.10 Microsatellite repeat analysis

To confirm identity of cell lines and subclones 9 highly polymorphic microsatellite markers were used (D1S1649, D2S434, D2S1384, D3S2427, D9S301, D9S934, D12S2070, D14S611, and D17S969). PCR conditions were as described in the Genome Database (GDB). One primer of each set was labelled with a fluorescent dye and PCR products were visualized by electrophoresis on a ABI 377 automatic sequencer. Data were analyzed with Genetyper software version 1.1.1 (Perkin Elmer, Foster City). Allele sizes were within expected range.

6.1.11 deposited clones

The sequences for human MRP-3, MRP-4, MRP-5 and for human and rat cMOAT are deposited at ECACC (European Collection of Cell Cultures, Salisbury, Wiltshire SP4 OJG, UK) under provisional numbers.

96010801-hu-MRP3 #96A
96010802-hu-MRP5 #97
96010803-hu-MRP4 #38089
96010804-hu-cMOAT #33A
96010805-hu-MRP3 #20.11
96010806-hu-MRP5 #101
96010807-hu-MRP5 #104
96010808-hu-MRP5 #105
96010809-hu-MRP3 #20.1
96010810-rat-cMOAT
96010811-hu-MRP3 #97F

6.2 Results

6.2.1 Database search for *MRP* homologs

We searched human EST databases (dbEST, TIGR) for *MRP* homologs other than *MRP1* and *cMOAT*. Alignment and comparison of EST sequences with homology specific to the 3'-terminal ends of *MRP1* and *cMOAT*, including the coding sequence for the second ATP-binding domain, revealed that there are at least 4 more *MRP* homologs expressed in humans. One of these homologs is the human sulfonylurea receptor (*SUR*) gene (73). The other three *MRP* homologs had not been identified before, and were designated *MRP3*, *MRP4*, and *MRP5*.

6.2.2 Cloning and sequencing of *MRP3*, *MRP4*, and *MRP5* cDNA

Additional cDNA clones for *MRP3* and *MRP5* were isolated from a human liver and a fetal brain cDNA library, respectively. *MRP3* and *MRP5* cDNA clones were sequenced as well as the *MRP4* cDNA clone obtained from the I.M.A.G.E. consortium. Both *MRP3* and *MRP5* encode four domain proteins, i.e. proteins with two ATP-binding domains and two domains with transmembrane regions (M.K. and J. Wijnholds, unpublished results). More sequence data will determine whether this is also the case for *MRP4*. Figure 14 shows the protein alignment for the COOH-terminal ends of the various members

of the human MRP family and human SUR. The alignment includes the Walker A and B motifs and the signature sequence of the second ATP-binding domain. The percentages of homology for the COOH-terminal 124 amino acids are shown in table 1. The highest homology is found between MRP1 and MRP3 (86% similarity) and the lowest between SUR and any of the MRPs (≤ 69% similarity).

6.2.3 Chromosome localization of MRP3, MRP4, and MRP5

The *MRP1* gene has been mapped to chromosome 16 at band p13.13-13.12 (5) and recently the *cMOAT* gene to chromosome 10, band q24 (52, 74). We mapped the other *MRP* homologs on the Gb4RH and StG3 radiation hybrid mapping panels, using *MRP3*, *MRP4*, or *MRP5* specific primers. *MRP3*, *MRP4*, and *MRP5*, are located on chromosomes 17, 13, and 3, respectively. The most closely linked markers were D17S797 (Gb4RH) and D17S1989 (StG3) for *MRP3*, WI-9265 (Gb4RH) and D13S281 (StG3) for *MRP4*, and WI-6365 (Gb4RH) and D3S4205 (StG3) for *MRP5*. These results are consistent between the radiation hybrid mapping panels and demonstrate that the new *MRP* homologs are indeed new genes, and not splice variants of *MRP1* or *cMOAT*.

6.2.4 Human tissue distribution of *cMOAT*, *MRP3*, *MRP4*, and *MRP5* RNA

RNase protections were performed to determine the tissues that express *cMOAT* and *MRP3*, *MRP4*, and *MRP5*. The results are summarized in table 2. Both *cMOAT* and *MRP3* are highly expressed in liver, and to a lower extent also in duodenum. Low expression of *cMOAT* was found in kidney and peripheral nerve. For *MRP3*, substantial expression, similar to expression in duodenum, was also detected in colon and adrenal gland. *MRP4* is expressed at a low level in only a few tissues tested. *MRP5* RNA was detected in substantial amounts in every tissue tested, with relatively high expression in skeletal muscle and brain.

6.2.5 Expression of *MRP* homologs in resistant cell lines

In view of their homology with *MRP1*, *cMOAT* and the three new *MRP* homologs are believed to encode transporter proteins involved in drug resistance. We therefore screened a large set of human cell lines derived from various tissues and their resistant sublines selected with either doxorubicin, cisplatin, tetraplatin, or $CdCl_2$. Only resistant lines showing decreased cellular accumulation of drugs were analyzed. All cell lines were analyzed by RNase protection for levels of *MDR1*, *MRP1*, *cMOAT*, *MRP3*, *MRP4*, *MRP5*, and actin RNA. The results are summarized in tables 3 and 4, and an example of each probe is shown in Fig. 15.

High *MDR1* overexpression was detected only in two sublines of the human non-small-cell lung cancer cell line SW1573/S1, both selected for high level doxorubicin resistance (2R160 and 1R500). The low level of *MDR1* RNA in the other cell lines is not remarkable as most of the cell lines selected for our panel were known to have a non-Pgp MDR phenotype. Low *MDR1* overexpression was found in the 2R120, a subline of the SW1573/S1, and in three cisplatin selected sublines of the bladder carcinoma cell line T24. Interestingly, a decrease rather than an increase in *MDR1* RNA was seen in two cisplatin selected sublines of the ovarium carcinoma cell line 2008 (table 4). This phenomenon has been reported earlier in the SW1573/S1 sublines 1R50b, 2R50, and 3R80, selected for low level doxorubicin resistance (58, 59; Table 3).

MRP1 RNA is highly overexpressed in the four non-Pgp MDR cell lines GLC_4/ADR, MOR/R, COR-L23/R, and HL60/ADR, all selected for high level doxorubicin resistance (7, 75, 76). The doxorubicin selected cell lines, derived from the SW1573/S1 cell line, showed no or only a minor increase in *MRP1* RNA, as reported before (7, 77). In the cell lines, selected for cisplatin resistance, we detected no major changes in *MRP1* RNA. Only in two sublines of the T24 cell line, T24/DDP7 and T24/DDP10, and in HCT8/DDP, a subline of

the colon carcinoma HCT8 cell line, a slight (less than 2 fold) increase in *MRP1* RNA was found.

Expression of *cMOAT* varied greatly between the cell lines. Most parental cell lines did not express *cMOAT* or at very low levels. Only the MOR/P and the KB-3-1 parental cell lines showed substantial *cMOAT* RNA levels. Overexpression of *cMOAT* was found in several doxorubicin-resistant sublines of SW1573/S1 (30.3M, 1R50b, 2R120, 2R160, and 1R500), and some cisplatin selected cell lines (2008/C13*5.25, 2008/A, A2780/DDP, and HCT8/DDP).

Similar to *cMOAT*, most parental cell lines either did not express *MRP3* or only at very low levels. The only two parental cell lines, which show high expression of *MRP3*, the MOR/P and the KB-3-1, also show high expression of *cMOAT*. Overexpression of *MRP3* in resistant lines was only found in several doxorubicin-resistant sublines of the SW1573/S1 cell line and the cisplatin resistant HCT8/DDP cell line.

MRP4 is expressed only at low or very low levels in the cell lines we analyzed and no overexpression of *MRP4* was detected in resistant sublines.

MRP5 is expressed in every cell line we analyzed, with the highest levels in MOR/P and 2008, but in none of the resistant sublines *MRP5* is highly overexpressed. Only in three cisplatin resistant cell lines, T24/DDP10, HCT8/DDP, and in the KCP-4(-), a minor increase in *MRP5* RNA was detected.

6.2.6 cMOAT protein in resistant cell lines

To investigate whether the increased *cMOAT* RNA levels in the resistant cell lines were accompanied by increased cMOAT protein levels, total cell lysates were tested on Western blot with the monoclonal antibodies M_2III-5 and M_2III-6, generated against amino acids 1340 to 1541 of the rat cmoat protein (48). To test the specificity for human proteins of the Mabs generated against rat cmoat, fusion proteins containing COOH-terminal ends of human cMOAT, MRP3, and MRP5, were made. Both cMOAT Mabs, M_2III-5 and M_2III-6,

recognize human cMOAT. M_2III-5 also reacts with the MRP5 fusion protein, and M_2III-6 also reacts with the MRP3 fusion protein. No cross-reaction was detected for both Mabs with MRP1 (data not shown).

Protein analysis of the cell lines with the cMOAT Mabs showed the presence of a 190-200 kDa protein in several lines (Fig. 16). Similar results were obtained with M_2III-5 and with M_2III-6 (not shown), indicating that the protein detected is cMOAT. The level of cMOAT protein in each cell line correlated very well with the level of *cMOAT* RNA, even for the cell lines with only a marginal increase in *cMOAT* RNA, such as the 2008/C13*5.25 and the 2008/A. The only exception was the cisplatin resistant subline of KB-3-1. KCP-4(-). The Western blot shows that the cMOAT protein level was about 2-3 fold higher in the KCP-4(-) cell line than in the KB-3-1, whereas the RNA levels were the same in parental and resistant cells. Mab M_2III-5 also reacts with MRP5 and MRP5 RNA is raised in the KCP-4(-) cells, but a similar result was obtained with Mab M_2III-6 which does not cross-react with MRP5.

All cell lines with no or only very low levels of *cMOAT* RNA also contained no detectable cMOAT protein (Fig. 16). The small amount of cMOAT detected in the parental A2780 cell line migrated faster in the gel than the cMOAT protein present in the cisplatin resistant A2780/DDP cell line, or the protein detected in the HCT8, HCT8/DDP, KB-3-1, and KCP-4(-) cells. The varying mobility of cMOAT in the gel could be caused by different degrees of post-translational modification of cMOAT protein in each cell line, as we have observed for MRP1 (77; M.K. unpublished results), but this needs to be verified.

6.2.7 Glutathione assays

In view of the proposed role of cMOAT as a GS-X pump, intracellular GSH levels were measured for the cell lines in table 4. GSH levels were elevated in all resistant cell

lines (Table 4), and were not detectably different in cells cultured with or without drugs (data not shown).

6.2.8 Drug resistance of the cell lines analyzed

To determine whether there is a correlation between the elevation of expression of transporters and resistance pattern, we have extended the existing information on these cell lines with a more complete survey of resistance against either cisplatin or doxorubicin (Table 5). Interestingly, all the doxorubicin selected SW1573 cell lines with overexpression of *cMOAT* are also cross-resistant against cisplatin, and the level of *cMOAT* expression correlates quite well with the level of cisplatin resistance (Tables 3 and 5). Cytotoxicity analysis of the KCP-4(-) cell line showed that the IC_{50} for cisplatin for this cell line was much lower than reported (700 nM, RF 1.8 [table 5] instead of 25.000 nM, RF 62.5 [37]), suggesting that this cell line was a revertant or contaminated with another low-level-cisplatin resistant cell line. When these KCP-4(-) cells were cultured in the presence of 6.7 μM cisplatin, more than 99% of the cells died. The surviving population, KCP-4(+), was highly cisplatin resistant again (IC_{50} 22.400, RF 59 [table 5]), but did not express *cMOAT* anymore (Fig. 16). Microsatellite repeat analysis showed that both cell lines, KCP-4(-) and KCP-4(+), were derived from the parental KB-3-1, indicating that the KCP-4(-) is most likely a revertant.

All cell lines selected for resistance against cisplatin, tetraplatin or $CdCl_2$ are not cross-resistant against doxorubicin (Table 5), with two exceptions: the KCP-4(-) cell line and the PXN94/tetR cell line. Cross-resistance did not correlate with *cMOAT* expression.

6.3. Discussion.

6.3.1.The *MRP* gene family

Our database search of expressed sequence tags has revealed that at least five homologs of *MRP1* are expressed in man.

cMOAT or *MRP2* encodes the major organic anion transporter in the canalicular membrane of hepatocytes (48-52, 55). The product of another homolog, *SUR*, plays a role in the regulation of insulin secretion (73). The other three homologs, *MRP3-5*, are all more related to MRP1 than SUR (Table 1). Identity is highest between MRP1 and MRP3 (75%). Since the region taken for comparison is small and one of the most conserved parts of the protein, the overall identity between the MRP homologs will probably be lower than the percentages in Table 1.

The *MRP* homologs MRP3-5 are all located on other chromosomes than *MRP1* and *cMOAT*. This confirms that *MRP3*, *MRP4*, and *MRP5* are not alternative splice products of *MRP1* or *cMOAT*. Klugbauer and Hofmann (78) recently cloned another ABC transporter (*ABC-C*), located in the same chromosomal band as *MRP1*, but this is not a *MRP* homolog, because the identity between these two proteins is only 18%. After our work was completed Allikmets *et al.* (79) reported the identification of 21 new ABC genes also based on a search of the human EST database and they mapped the identified partial sequences.

6.3.2 Physiological functions of the MRP family members

The physiological role of these new MRP proteins is probably a role in cellular detoxification processes by exporting GSH *S*-conjugates or other organic anions. GSH *S*-conjugate carriers have been described in many mammalian cells, including liver, heart, lung, and mast cells and erythrocytes (1B, 80). Kinetic studies indicate that both liver canaliculi and erythrocytes contain two different ATP-dependent transport activities for organic anions (81-84). cMOAT is localized in the canalicular membranes of hepatocytes and the absence of this protein in the TR$^-$ rats as well as in a patient with the Dubin-Johnson syndrome shows a role for the cmoat/cMOAT proteins in the transport of non-bile acidic organic compounds from liver to bile (48, 49, 55). The other ATP-dependent transport activity in liver canaliculi, responsible for transport of bile acids from liver to bile is not attributable to cMOAT, because studies with TR$^-$ rats and Dubin-Johnson patients showed that bile acid transport was not affected (1B).

Two other congenital liver diseases characterized by a conjugated hyperbilirubinemia, like the Dubin-Johnson syndrome, are Benign Recurrent Intrahepatic Chclestasis (BRIC) and Progressive Familial Intrahepatic Cholestasis (PFIC or Byler disease) (85, 86). The clinical and biochemical features of BRIC and PFIC are suggestive of a defect in primary bile acid secretion (87, 88). BRIC and PFIC have both been mapped to the same region on chromosome 18, 18q21-q22 (89, 90).

In view of the high expression of *MRP3* in the liver (Table 2), *MRP3* may be the bile salt transporter. Since none of the human ABC transporter genes identified thus far maps to chromosome 18 (79; this study) it is unlikely that BRIC/PFIC is caused by a defect in a readily recognizable ABC transporter gene.

GS-X activity has also been found in erythrocytes. Several studies have shown that human and rat erythrocytes

contain a low- and a high-affinity S-(2,4-dinitrophenyl)-glutathione (DNP-SG) transporter (84, 91, 92). The high-affinity DNP-SG transporter is most likely MRP1, since the presence of this protein and its binding to LTC_4 have been shown for erythrocytes (93, 94). The other transporter with low affinity for DNP-SG but high affinity for glucuronides and mercapturates (84) is not cMOAT or the bile salt transporter, because (i) no major alterations in DNP-SG transport in erythrocytes from TR$^-$ rats and Dubin-Johnson patients were detected (1B), and (ii) erythrocytes transport DNP-SG and GSSG but no bile salts (83). This second transporter may be encoded by one of the other MRP homologs.

6.3.3 Expression of *MRP* homologs in resistant cell lines

We screened a large set of cell lines and their resistant sublines to see whether *MRP1*, *cMOAT* or one of the other *MRP* homologs is overexpressed. *MRP4* was not overexpressed in any of the lines. *MRP3* RNA was only found to be elevated in the cisplatin resistant HCT8/DDP cell line and several SW1573/S1 sublines selected for doxorubicin resistance. However, overexpression did not correlate with the level of doxorubicin resistance. For *MRP5* low overexpression was found in three cell lines selected for cisplatin resistance (T24/DDP10, HCT8/DDP, and KCP-4(-); Table 4), but many other cisplatin selected cell lines showed no overexpression.

Table 3 shows that the classical non-Pgp cell lines selected for high doxorubicin resistance and known to highly overexpress the *MRP1* gene, do not significantly overexpress other members of the *MRP* family. This is compatible with the interpretation that MRP1 is the transporter responsible for MDR in these cell lines. In the non-Pgp derivatives of the SW1573/S1 cell line presented in Table 3 a more complex situation is found and the contribution of MRP1, cMOAT, MRP3, and the major vault protein, also present at increased levels in some of these cell lines (95), remains to be sorted out.

6.3.4 The involvement of organic anion transporters in cisplatin resistance

Whereas P-glycoproteins do not transport small or highly charged molecules, organic anion transporters, such as MRP1 and cMOAT have been speculatively linked to resistance to oxyanions (arsenite, antimonite) and cisplatin. These compounds can form complexes with GSH and there is now considerable evidence that these complexes are substrates for organic anion transporters. Resistance caused by increased export of these complexes is bound to be complex, as pointed out by Ishikawa (80) and by us (6b, 19, 95). Increased levels of pump or GSH, increased GSH synthesis, or a combination may be required depending on the rate limiting step in drug export.

In the protozoal parasite *Leishmania*, resistance to arsenite and antimonite can be associated with both a 40-fold increase in the *Leishmania* GSH homolog trypanothione (97) and an increase in the MRP-related ABC-transporter PgpA (98). Cancer cells selected for high levels of cisplatin may sometimes also contain extremely high concentrations of GSH (99) and the GSH synthesis in these cells is upregulated (99-101). All of the cisplatin resistant cell lines studied by us have elevated GSH levels as well, albeit not as high as the cell lines isolated by Godwin *et al.* (99). In contrast to published data, we also find raised GSH levels in the T24 sublines, the GCT27cisR, and the PXN94/tetR cell lines (42, 44, 45). We find no clear correlation, however, between the degree of cisplatin resistance and GSH levels, as observed by Godwin *et al.* (99). Moreover, all the cell lines studied by us show a decreased accumulation of cisplatin and an organic ion pump may therefore be involved in resistance.

Ishikawa *et al.* (36) showed that *MRP1* is overexpressed in the cisplatin resistant human leukemia cell line HL60/R-CP. They concluded that an increased GSH synthesis in combination with raised MRP1 levels can cause cisplatin

resistance. Active cisplatin efflux has been described in three of the cell lines in Table 4: KCP-4, A2780/DDP, and HCT8/DDP (37-39, 102). The ATP-dependent efflux was inhibited by DNP-SG, indicating that it was catalyzed by a GS-X pump. In addition, the membrane vesicles of the KCP-4 cell line were shown to catalyze an increased uptake of LTC_4 (37, 38), known to be the substrate with the highest affinity for MRP1. However, data from these papers and our study show that *MRP1* is not overexpressed in these cisplatin resistant cell lines, suggesting that MRP1 is not the major pump responsible for cisplatin resistance. This is supported by transfection studies with *MRP1*, which showed no cisplatin resistance of the transfected cells (28, 17). Nevertheless, it remains possible that transport of cisplatin conjugates by MRP1 is efficient and that the low levels of MRP1 present in parental cells suffice for resistance, if formation of cisplatin conjugates in resistant cells is increased, e.g. by an increase in GSH synthesis.

An organic anion pump that is important in cisplatin resistance is cMOAT. Especially striking is the correlation between cisplatin resistance and *cMOAT* expression in the non-Pgp MDR cell lines derived from the SW1573/S1 cell line (Table 5). These lines were selected for doxorubicin resistance and it is therefore unlikely that other mechanisms of cisplatin resistance are activated in these lines. It should be noted, that these non-Pgp MDR lines, selected for low level doxorubicin resistance, contain multiple alterations in the expression of ABC-transporters. Besides upregulation of *MRP1*, *cMOAT*, and *MRP3* (Table 3), down-regulation of *MDR1* has occurred in these lines (59; this study, Table 3).

Some other cisplatin-resistant lines contain increased levels of *cMOAT* as well, notably 2008/C13*5.25, 2008/A, A2780/DDP, and HCT8/DDP (Fig. 3, Table 4 and 5).

The combination of cisplatin with doxorubicin resistance in resistant cell lines has been reported before (100, 103) and is also present in two other platin selected lines,

studied here, PXN94/tetR and KCP-4(-) (Table 5). All other platin-selected lines in Table 5 are doxorubicin sensitive, however. The substrate specificity of the organic anion pumps in the liver canalicular membrane (cMOAT) and in erythrocytes (presumably mainly if not exclusively MRP1) is very similar (91). We therefore expect both pumps to confer similar resistance spectra. We have recently succeeded in obtaining stably transfected kidney cells in which cMOAT is properly routed to the plasma membrane (R. Evers, M.K., and P.B., unpublished). These cells should allow a direct test of the drug resistance spectrum that can be associated with *cMOAT* overexpression.

Overexpression of *cMOAT* in cisplatin resistant cell lines was recently also reported by Taniguchi *et al.* (52). However, in contrast to our results (Table 4) they detected raised *cMOAT* RNA levels in the KCP-4 and T24/DDP10 cell lines. We do not find this. The level of *cMOAT* RNA was even decreased in the highly cisplatin resistant KCP-4(+) cells, and in the T24/DDP10 cell line *cMOAT* RNA is hardly detectable by RNase protection. We also detect no cMOAT protein in these cell lines (Fig. 16). Cross-hybridization of the *cMOAT* probe used by Taniguchi *et al.* (52), which contains the coding sequence of the first ATP-binding domain, with RNA transcribed from the other *MRP* homologs might explain the discrepancy. This underlines the importance of the use of gene specific probes to determine expression of *MRP* homologs.

In conclusion, our data and those recently published by Ishikawa *et al.* (35, 36), Fujii *et al.* (37, 38), Goto *et al.* (39), Chuman *et al.* (102), and Taniguchi *et al.* (52) provide evidence that an organic anion pump, notably cMOAT, contributes to cisplatin resistance by exporting the cisplatin-GSH complex. Elevated GSH levels and synthesis may be required to drive formation of the complex if contact with cisplatin is extended, as is usually the case for cell lines selected for resistance *in vitro*.

LEGENDS TO THE FIGURES.

Figure 1a. cDNA sequence of human cMOAT

Figure 1b. cDNA sequence of rat cMOAT

Figure 2. (A) Northern blot analysis of 2 µg poly(A)$^+$ RNA from Wistar rat tissues hybridized to a 1-kb *Hind*III/*Ava*II cDNA fragment of *cmoat*. RNA was analyzed as described in (the experimental part. Prolonged exposure of the film revealed no detectable expression in other tissues then kidney, duodenum, and ileum. (**B**) Northern blot analysis of 2 µg of poly(A)$^+$ RNA from Wistar and TR$^-$ rat liver and hepatocytes hybridized with the same probe as described in (A). The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) signal is shown at the bottom. Molecular size standards are indicated at the right.

Figure 3. Immunoblot analysis of cmoat and P-glycoprotein in canalicular and basolateral membrane fractions of Wistar and TR$^-$ rat hepatocytes. Lane 1, Wistar basolateral membranes; Lane 2, Wistar canalicular membranes; Lane 3, TR$^-$ basolateral membranes; Lane 4, TR$^-$ canalicular membranes. Upper panel: the blot was incubated with the monoclonal antibody M_2 III-5 directed to cmoat (27). This antibody did not crossreact with the hMRP1 protein as tested in total lysates from the MRP-overexpressing cell line GLC4/ADR (20). Lower panel: Immunodetection of P-glycoproteins with the Mab C219 in the same membrane preparations. The 150-kD P-glycoproteins are exclusively expressed in canalicular membranes (22). Differential staining of the two fractions demonstrates the separation of the two membrane domains with slight contamination of the basolateral fraction by canalicular membranes. Molecular weight markers are indicated.

Figure 4 Deduced amino acid sequence of the rat cMOAT and alignment with the deduced 70 amino acid sequence of the translated 213-bp putative rat *mrp1* cDNA.

Figure 5. Transport of GS-DNP in COS-7 cells transiently transfected with rat cMOAT expression constructs. Closed

circles represent cells transfected with pSVK3-rat cMOAT. Open circles represent cells that have been transfected with a pSVK3 construct with the rat cMOAT cDNA in the reverse orientation and serves as a negative control. The results depicted are the mean of three measurements.

Figure 6. Transport of GS-DNP in membrane vesicles prepared from COS-7 cells transiently transfected with rat cMOAT expression constructs in the presence or absence of an ATP regenerating system. Closed squares represent cells transfected with pSVK3-rat cMOAT. Open squares represent cells that have been transfected with a pSVK3 construct with the rat cMOAT cDNA in the reverse orientation and serves as a negative control. The results depicted are the mean of three measurements.

Figure 7. Human cMOAT expression in crude lysates from MDCKII derived transfectants. 2 or 20 mg of total protein was size fractionated in a 7.5% polyacrylamide gel containing 0.1% (wt/vol) SDS. After electroblotting, human cMOAT protein was visualized by staining with mAB M_2-III-6. Protein antibody interaction was detected using the Amersham enhanced chemiluminescence kit (ECL). Lane 1,2 MDCKII cells; lane 3,4 human cMOAT expressing clone MDCKII-216; lane 5,6 human cMOAT expressing clone MDCKII-217. In lanes 1,3 and 5 two micrograms of total protein were loaded and in lanes 2,4 and 6 20 micrograms were loaded.

Figure 8. Detection of human cMOAT in MDCKII monolayers by confocal laser-scanning microscopy. A. Indirect immunofluorescence (FITC) picture with mAb M_2-III-6 on MDCKII-217 cells. Nucleic acids were detected using propidiumiodide (red signal). Top view of the cell layer is shown. B. Optical section perpendicular to the plane of the cell layer.

Figure 9. Export of GS-DNP from MDCK-II, MDCKII-216, and MDCKII-217 cells. Cells were incubated with [^{14}C]CDNB (2 mM) in both the apical and basal compartments. Samples were taken at t = 1, 3, 6, 12, and 20 min from both compartments and extracted with ethylacetate. The amount of [^{14}C]DNP-GS

excreted (in pmol per 2 ml) was measured and plotted. All experiments were done in duplicate and repeated at least twice. Variation between measurements was below 10%. Dotted line: transport to the basal compartment. Continuous line: transport to the apical compartment.

Figure 10. Deduced amino acid sequence of human cMOAT. Predicted transmembrane regions are underlined. Walker A, B, and signature sequence are doubly underlined. Predicted N-glycosylation sites conserved in other cMOAT proteins (rat, rabbit) and MRP1 proteins (human, mouse) are indicated with triple asterisks. The triangle indicates the location (amino acid 1066) at which a stop codon is introduced by a C to T transition in DJS *cMOAT*.

Figure 11. Immunohistochemical detection of the cMOAT protein in human and rat liver using monoclonal antibody M_2III-6. Sections of a normal human liver (*A*) and normal rat liver (*B*), which demonstrate the exclusive canalicular localization of the protein. In liver sections of the DJS patient (*C*) and the TR$^-$ rat (*D*), no canalicular staining is observed. Magnifications are 20 x (*A, C*), and 100 x (*B, D*).

Figure 12. Part of the *cMOAT* cDNA sequence encompassing the mutation which results in the absence of the functional protein in the patient. The normal sequence is depicted on the right. The arrow indicates the site of the mutation at codon 1066. This codon normally encodes an arginine residue (CGA), but is changed into a stop-codon (TGA) in the patient. The mutation of C to T eliminates the recognition site for the restriction enzyme *Taq*I (5'-T<u>C</u>GA-3').

Figure 13. *Taq*I digest of a part of the *cMOAT* cDNA that was obtained with primer combination forwardIV/reverseIV. Lane 1 represents healthy control-, lane 2 the patient cDNA digest. Molecular size markers are indicated on the left in kilo base pairs.

Figure 14

Protein alignment of COOH-terminal ends of the five human MRP homologs and human SUR. The alignment was performed with the PILEUP program of GCG (48). The GenBank accession

numbers for the proteins used in this comparison are the following: MRP1 - L05628, cMOAT/MRP2 - U49248, MRP3 - U83659, MRP4 -U83660, MRP5 - U83661, SUR - L78207. The nucleotide binding domain specific signature sequence and the Walker A and B motifs are shown in bold. Asterisks above the alignment indicate identical amino acids in at least four of the five MRP proteins.

Figure 15

RNase protection assays of RNA transcript levels of *MDR1*, *MRP1*, *cMOAT* (*MRP2*), *MRP3*, *MRP4*, and *MRP5* in the human non-small-cell lung cancer cell line SW1573/S1 and its doxorubicin selected subline 30.3M. 10 µg total cytoplasmic RNA from each cell line was used per probe. The positions of the protected fragments of *MDR1*, *MRP1-5*, and t-actin are indicated.

Figure 16

Immunoblot detection of cMOAT protein in the cell lines analyzed in this paper. Total cell lysates were size fractionated (40 µg per lane) in a 7.5% polyacrylamide gel containing 0.5% SDS. The fractionated proteins were transferred to a nitrocellulose membrane, and cMOAT protein was detected by incubation with monoclonal antibody M_2III-5. The size (kDa) and position of molecular weight markers are indicated.

Figure 17 A/B.

Nucleotide sequence and amino acid sequence of MRP3.

Figure 18.

Partial MRP4 sequence.

Figure 19 A/B

MRP 5 sequences.

Table 1

Homology between the COOH-terminal 124 amino acids of the five human MRP homologs and human SUR. Percentages of identity and similarity were determined using the BESTFIT program of GCG (48).

Table 2

Levels of RNA transcripts of *MRP1*, *cMOAT* (*MRP2*), *MRP3*, *MRP4*, and *MRP5* in human tissues. RNA expression levels were determined by RNase protection assays with 10 µg total RNA from various human tissues per probe. Expression of t-actin was taken as control for total RNA input. Data for *MRP1* RNA levels are from Zaman *et al.* (52). The relative expression level is indicated by filled circles, very low or undetectable RNA levels by open circles. nd = not determined.

Table 3

Characteristics of the doxorubicin-selected cell lines analyzed in this paper. Resistant cell lines were selected by chronically exposing them to the concentrations of doxorubicin as shown. RNA levels were determined as in Figure 2. The relative expression level is indicated by filled circles, very low expression by _, and undetectable RNA levels by open circles.

Table 4

Characteristics of cell lines selected for resistance to cisplatin, tetraplatin or $CdCl_2$. Resistant cell lines were selected by chronically exposing them to the concentrations of drugs as shown. Only A2780/DDP and HCT8/DDP were selected by challenging them 1 h weekly with 50 µM cisplatin. RNA levels were determined as in Figure 2. The relative expression level is indicated by filled circles, very low expression by _, and undetectable RNA levels by open circles. Data for total intracellular glutathione concentrations were obtained from three independently isolated cell extracts assayed in three independent experiments using the recycling method of Tietze (56) and presented as the mean GSH ± SD.

Table 5

IC_{50} values and relative resistance factors (RF) of the cell lines analyzed for cisplatin and doxorubicin. IC_{50} data were obtained from clonogenic survival assays with continuous exposure to drugs. The relative resistance factor was

determined by dividing the IC_{50} of each resistant cell line by the IC_{50} of the corresponding parental cell line. Also shown are the levels of RNA transcripts of *MRP1* and *cMOAT*, taken from Table 3 and 4.

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Table 1

Homology between COOH-terminal 124 amino acids of human MRP homologs and human SUR (% similarity / % identity)

	MRP1	cMOAT	MRP3	MRP4	MRP5	HSUR
MRP1	100 / 100					
cMOAT	79 / 68	100 / 100				
MRP3	86 / 75	79 / 62	100 / 100			
MRP4	75 / 60	75 / 54	71 / 53	100 / 100		
MRP5	75 / 55	75 / 53	71 / 51	73 / 57	100 / 100	
HSUR	69 / 48	68 / 46	66 / 46	69 / 48	67 / 45	100 / 100

Table 2

Tissue distribution of human *MRP* gene transcripts

	MRP1	cMOAT	MRP3	MRP4	MRP5
Lung	●●●●	o	●	●	●●
Kidney	●●●	●	●	●	●●
Bladder	●●●●	o	●	●	●●
Spleen	●●●●	o	●	o	●●
Mamma	nd	o	o	nd	nd
Salivary gland	nd	o	o	o	●●
Thyroid	●●●●	o	o	o	●
Testis	●●●●	o	o	o	●●
Nerve	●	●	o	o	●●
Stomach	●●●	o	●	o	●●
Liver	o	●●●●	●●●●	o	●
Gall bladder	●●●	nd	nd	●	●●
Duodenum	●●	●●	●●●	nd	nd
Colon	●●●	o	●●●	o	●●
Adrenal gland	●●●●	o	●●●	o	●
Skeletal muscle	●●	o	o	o	●●●●
Heart	●	o	o	o	●●
Brain	●	o	o	o	●●●
Placenta	●●	o	o	o	●
Ovarium	●●	o	o	o	●
Pancreas	●	o	●	o	●
Tonsil	nd	o	●	●	●●

nd = not determined
o = no expression
● - ●●●● = low to high expression

Table 3

Characteristics of the doxorubicin-selected MDR cell lines analyzed

Cell line	Source	Drug used for selection	RNA levels					
			MDR1	MRP1	cMOAT	MRP3	MRP4	MRP5
S1	Non small cell lung cancer		⊕	•	⊕	⊕	•	•
30.3M		dox (30 nM)	⊕	••	•••	•••	•	•
1R50b		dox (50 nM)	o	•	••	•	•	•
2R50		dox (50 nM)	o	••	⊕	⊕	•	•
3R80		dox (80 nM)	o	•	⊕	••	•	•
2R120		dox (120 nM)	•	••	••	••	•	•
2R160		dox (160 nM)	••••	••	•••	•	•	•
1R500		dox (500 nM)	••••	•	••	⊕	•	•
COR-L23	Non small cell lung cancer		o	•	o	o	•	⊕
COR-L23/R		dox (368 nM)	o	••••	o	o	•	⊕
GLC_4	Small cell lung cancer		o	•	o	o	•	•
GLC_4/ADR		dox (1160 nM)	o	••••	o	o	•	•
MOR/P	Adeno lung carcinoma		o	•	••••	•••	•	•••
MOR/R		dox (368 nM)	o	••••	••••	•••	•	•••
HL60	Leukemia carcinoma		o	•	o	o	•	⊕
HL60/ADR		dox (186 nM)	o	••••	o	o	•	⊕

o = no expression

⊕ = very low expression

• - •••• = low to high expression

Table 4

Characteristics of the cell lines selected for resistance to cisplatin, tetraplatin or $CdCl_2$

Cell line	Source	Drug used for selection	RNA levels						GSH (nmol/mg protein)
			MDR1	MRP1	cMOAT	MRP3	MRP4	MRP5	
T24	Bladder carcinoma		o	•	⊕	⊕	•	•	11.4 ± 2.4
T24/DDP5		CDDP (3.3 μM)	•	•	⊕	⊕	•	•	26.8 ± 0.1
T24/DDP7		CDDP (4.7 μM)	•	••	⊕	⊕	•	•	65.5 ± 1.8
T24/DDP10		CDDP (6.7 μM)	•	••	⊕	⊕	•	••	59.5 ± 3.3
2008	Ovarium carcinoma		•	••	⊕	⊕	⊕	•••	33.5 ± 1.5
2008/C13*5.25		CDDP (5.0 μM)	o	••	•	⊕	⊕	•••	113.2 ± 16.6
2008/MT		$CdCl_2$ (25 μM)/ $ZnCl_2$ (200 μM)	•	••	⊕	⊕	⊕	•••	48.5 ± 5.4
2008/A		CDDP (0.5 μM)	o	••	•	⊕	⊕	•••	124.7 ± 18.6
A2780	Ovarium carcinoma		o	•	⊕	o	⊕	••	13.3 ± 0.9
A2780/DDP		CDDP (50.0 μM)	o	•	••••	o	⊕	••	26.5 ± 2.4
PXN94	Ovarium carcinoma		⊕	••	o	o	•	••	40.2 ± 5.6
PXN94/tetR		Tetraplatin (2.0 μM)	⊕	••	o	o	•	••	91.8 ± 8.8
HCT8	Colon carcinoma		••••	•	•	•	•	•	17.7 ± 2.2
HCT8.DDP		CDDP (50.0 μM)	••••	••	•••	••	•	••	70.2 ± 6.8
GCT27	Testis carcinoma		o	•	⊕	o	⊕	••	5.2 ± 0.2
GCT27cisR		CDDP (4.0 μM)	o	•	⊕	o	⊕	••	9.2 ± 3.5
KB-3-1	Epidermoid carcinoma		o	••	•••	•••	⊕	•	36.7 ± 0.9
KCP-4(-)		CDDP (23.3 μM)	o	••	•••	•••	⊕	••	72.7 ± 8.9
KCP-4(+)		CDDP (6.7 μM)	nd	nd	⊕	nd	nd	nd	158.4 ± 13.3

o = no expression
⊕ = very low expression
• . •••• = low to high expression
nd = not determined

Table 5

Cell line	Cisplatin		Doxorubicin		RNA levels	
	IC_{50} (nM)	RF	IC_{50} (nM)	RF	MRP1	cMOAT
S1	144	-	13.8	-	●	⊖
30.3M	500	3.5	51.1	3.7	●●	●●●
1R50b	194	1.5	84.2	6.1	●	●●
2R50	115	0.9	69.0	5.0	●●	⊖
3R80	nd	nd	75.9	5.5	●	⊕
2R120	313	2.2	345	25	●●	●●
2R160	600	4.2	1380	100	●●	●●●
1R500	260	1.7	3450	250	●	●●
T24	825	-	6.5	-	●	⊖
T24/DDP5	2200	2.7	5.5	0.8	●	⊖
T24/DDP7	1800	2.2	3.0	0.5	●●	⊖
T24/DDP10	8000	9.7	6.5	1.0	●●	⊖
2008	340	-	57.6	-	●●	⊕
2008/C13*5.25	3000	8.8	43.2	0.8	●●	●
2008/MT	210	0.6	54.4	0.9	●●	⊕
2008/A	680	2.0	36.8	0.6	●●	●
A2780	430	-	5.2	-	●	⊕
A2780/DDP	4300	10.0	6.2	1.2	●	●●●●
PXN94	580	-	4.2	-	●●	○
PXN94/tetR	2400	4.1	11.0	2.6	●●	○
HCT8	1900	-	90.0	-	●	●
HCT8/DDP	4800	2.5	95.0	1.1	●●	●●●
GCT27	288	-	3.0	-	●	⊕
GCT27cisR	2100	7.3	3.1	1.0	●	⊕
KB-3-1	380	-	11.0	-	●●	●●●
KCP-4(-)	700	1.8	43.0	3.9	●●	●●●
KCP-4(+)	22400	58.9	8.0	0.7	nd	⊕

CLAIMS

1. A nucleic acid comprising a sequence encoding at least a part of a member of a family of organic anion transporters, with the exclusion of mammalian Multidrug Resistance Associated Protein, said nucleic acid comprising at least a gene family specific fragment of one of the sequences of fig.1a or fig.1b or the complement thereof, or a sequence having at least 55%, preferably 70%, in particular 90% homology therewith.

2. A nucleic acid according to claim 1 encoding at least a part of a mammalian member of said family.

3. A nucleic acid according to claim 2 encoding at least a part of a human member of said family.

4. A nucleic acid and/or its complement having at least part of the sequence of fig.1A and encoding a protein having human Canalicular Multispecific Organic Anion Transport protein or similar activity or antigenicity.

5. A nucleic acid and/or its complement having at least part of the sequence of fig.1b and encoding a protein having rat Canalicular Multispecific Organic Anion Transport protein activity or antigenicity.

6. A nucleic acid encoding rat Canalicular Multispecific Organic Anion Transport protein or human Canalicular Multispecific Organic Anion Transport protein.

7. A vector comprising a nucleic acid according to anyone of the foregoing claims and suitable means for replication, transduction and/or expression of said nucleic acid.

8. A vector according to claim 7 further comprising a gene encoding a therapeutically beneficial protein.

9. A vector according to claim 7 or 8 further comprising a gene encoding glutathion elevating activity.

10. A vector according to claim 9 wherein the gene encodes at least a functional part of a gamma glutamyl cysteine synthetase or a UDP-glucuronosyltransferase.

11. A vector according to claim 7 wherein the therapeutically beneficial protein is another multidrugresistance related protein such as MDR1.

12. A cell comprising a nucleic acid or a vector according to anyone of the foregoing claims.

13. A cell according to claim 12 further comprising a vector encoding glutathion elevating activity.

14. A cell according to claim 12 or 13 provided with other resistance related proteins such as MDR1.

15 A method for providing cells with Canalicular Multispecific Organic Anion Transport protein activity, comprising transducing said cell with a nucleic acid or a vector according to anyone of claims 1-11.

16. A method for enhancing Canalicular Multispecific Organic Anion Transport protein activity of cells according to claim 15, comprising increasing the intracellular level of glutathion, glucuronide and/or sulphate.

17. A method for enhancing Canalicular Multispecific Organic Anion Transport protein activity of cells according to claim 15, comprising enhancing the conjugating capacity and/or the compelxing activity of said cell for sulphate, glutathion, glucuronide and the like.

18. A method for reducing Canalicular Multispecific Organic Anion Transport protein activity and/or the multidrug resistance of a cell comprising providing said cell with an antisense construct of a nucleic acid or a vector according to anyone of claims 1-11.

19. A method according to claim 18, further comprising providing the cell with an antisense construct derived from another multidrug resistance related protein such as MDR1.

20. A protein encoded by a nucleic acid according to anyone of claims 1-6 or obtainable by expression of a vector according to anyone of claims 7-11.

21. A protein having Canalicular Multispecific Organic Anion Transport protein activity or Canalicular Multispecific Organic Anion Transport protein specific

antigenicity comprising at least part of the sequence of fig.4 or another mammalian equivalent thereof.

22. Use of a nucleic acid according to anyone of claims 1-6 or a protein according to claim 20 or 21 in the diagnosis of Dubin-Johnson disease, Rotor disease or another disease involving Canalicular Multispecific Organic Anion Transport protein.

23. Use of a nucleic acid according to anyone of claims 1-6 or a protein according to claim 20 or 21 in the treatment of Dubin-Johnson disease, Rotor disease or another disease involving Canalicular Multispecific Organic Anion Transport protein.

24. Use of a nucleic acid according to anyone of claims 1-6 as a selectable marker gene.

```
   1  AGGATAATTC CTGTTCCACT TTCTTTGATG AAACAAGTAA AGAAGAAACA
  51  ACACAATCAT ATTAATAgAA GAGTctTCGt TCCAGACgCA GtCCAGGAAT
 101  CATGCTGGAG AAgTTCTGCA ACTcTACTTT tTGGAATTCc TCATTCcTgg
 151  ACAGTCCGGA GGCAgAcCTG cCacTTTGTt TtGAGCAAAc TGTTcTGGTG
 201  TGGATTCCcT TGGGCTTCcT ATGGCTCCTG GCCCCctGgC agcTTcTCcA
 251  cGtGtAtAAA tCCaggACCA AGaGATCcTc TACCACCAAA cTctaTcTTG
 301  cTAaGCaGGT ATTcGttGgT tTtCttcTTA TtcTAgCAgc CATAGAGCtG
 351  GCCCTTGTAC TCACAGaAGA CTCTGGACAA GCCACAGTCC cTGCTGTtCG
 401  ATATACCAAT CCAAGcCTcT ACCTAGgCAC AtGGcTCCTG GTTTTGCTGA
 451  TCCAATACaG cAGACAATGG TGTGTACAGA AAAACTCCTG GTtCCTGtCC
 501  cTATTcTGGA TTCTCTcGAT ACTCtGtGGC ACTTTcCAaT TTCAGACTCt
 551  gATccGGAca CTCTTACAGG GTGACAAtTc TAATCTAGCc TACTCcTGcC
 601  TGTTcTTCAt CtCcTAcGGa tTcCaGATCc TGATCCTGAT CtTTTCAGCA
 651  TTTTCAGAAA ATAATGAgTC ATCAAATAAT CCATCATCCA TAGCTTCAtT
 701  CCTGAgTAgC ATTACCTACA GCTGGTATGA CAgCaTCATT CTGAAAgGcT
 751  ACaAgCgTcC TCTGACACTc gAgGATgTcT GGGAAgttGA TGAAgAgATg
 801  AAAACCaAGA CATTAGTGAG CAAGTTTGaa ACGCACATGA AGAGAGAGCT
 851  GCAGAAAGCC AGGCGGGCAC TCCAGAGACG GCAGGAGAAG AGCTCCCAGC
 901  AGAACTCTGG AgCCAGGCTG CCTGGCTTGA ACAAGAATCA GAGTCAAAGC
 951  CAAGATGCCC TTGTCCTGGA AGATGTTGAA AAGAAAAAAA AGAAgTCTGG
1001  GACCAAAAAA GATgTTCCAA AATCCTGGTT GATGAAgGCt CTGTTCAAAA
1051  CTTTCTACAT GGTGCTCCTG AAATCaTTCC TACTGAAgCT AGTGAATGAC
1101  aTCTTCACgT TTGTGAGTCC TCAgCTGCTG AAAtTGCTGA TCTCCtTTGC
1151  AAGTGAcCGt GACaCATAtT TGTGGATTGG ATATCTCTGT GCaATCCTCT
1201  TAtTCaCTGC GGcTCTCATT CAGTCtTTCt GCCTTCAgTG TTaTTTCCAA
1251  CTGTGCTTCA AGCTGGGTGT AAAAgTACGg ACAGCtATCa tgGcTTcTGT
1301  ATATAAGAAG GCATTGACCC TAtCCAACTT GGCCaGGAAG GAGTACACCG
1351  tTGGAGAAAC AGTGAACCTG ATGTCTGTGG ATGCCCAGAA GcTCATGGAT
1401  GTGACCAACT TCATGCACAT GCTGTGGTCA AGTGTTcTAC AGATTGTCTT
```

FIG. 1a

1451 ATCTATCTTC TTCCTAtGGA GAGAGTTGGG ACCCTCAGTC TTAGCAGGTG

1501 TTGGGGTGAT GGTGCTTGTA ATCCCAATTA ATGCGATACT GTCCACCAAG

1551 AGTAAGACCA TTCAGGTCAA AAATATGaAG AATAAAGACA AACGTTTAAA

1601 GATCATGAAT GAGATTCTTA GTGGAATCAA GATCCTGAAA TATTTTGCCT

1651 GGGAACCTTC ATTCAGAGAC CAAGTACAAA ACCTCCGGAA GAAAGAGCTC

1701 AAGAACCTGC TGGCCTTTAG TCAAcTACAG TGTGTAgTAA TATTcGTCTT

1751 CCAGTTAACT CCAGTcCTGG TATCTGTGGT CACATTTTCT GTTTATGTCC

1801 TGGTGGATAg CAACAATATT TtGGATGCAC AAAAGGCCTT CACCTCCATT

1851 ACCCTCTTCA ATATCCTGCG CTTCCCCCTG AGCATGCTTC CCATGATGAT

1901 CTCCTCCATG CTCCAGGCCA GTGTTTCCAC AGAGCGGCTA GAGAAGTACT

1951 TGGGAGGGGA TGACTTGGAC ACATCTGCCA TTCGACATGA CTGCAATTTT

2001 GACAAAGCCA TGCAGTTTTC TGAGGCCTCC TTTACCTGGG AACATGATTC

2051 GGAAGCCACA GTCCGAGATG TGAACCTGGA CATTATGGCA GGCCAACTTG

2101 TGGCTGTGAT AGGCCCTGTC GGCTCTGGGA AATCCTCCTT GATATCAGCC

2151 ATGCTGGGAG AAATGGAAAA TGTCCACGGG CACATCACCA TCAAGGGCAC

2201 CACTGCCTAT GTCCCACAGC AGTCCTGGAT TCAGAATGGC ACCATAAAGG

2251 ACAACATCCT TTTTGGAACA GAGTTTAATG AAAAGAGGTA CCAGCAAGTA

2301 CTGGAGGCCT GTGCTCTCCT CCCAGACTTG GAAATGCTGC CTGGAGGAGA

2351 TTTGGCTGAG ATTGGAGAGA AGGGTATAAA TCTTAGTGGG GGTCAGAAGC

2401 AGCGGATCAG CCTGGCCAGA GCTACCTACC AAAATTTAGA CATCTATCTT

2451 CTAGATGACC CCCTGTCTGC AGTGGATGCT CATGTAGGAA AACATATTTT

2501 TAATAAGGTC TTGGGCCCCA ATGGCCTGTT GAAAGGCAAG ACTCGACTCT

2551 TGGTTACACA TAGCATGCAC TTTCTTCCTC AAGTGGATGA GATTGTAGTT

2601 CTGGGGAATG GAACAATTGT AGAGAAAGGA TCCTACAGTG CTCTCCTGGC

2651 CAAAAAAGGA GAGTTTGCTA AGAATCTGAA GACATTTCTA AGACATACAG

2701 GCCCTGAAGA GGAAGCCACA GTCCATGATG GCAGTGAAGA AGAAGACGAT

2751 GACTATGGGC TGATATCCAG TGTGGAAGAG ATCCCCGAAG ATGCAGCCTC

2801 CATAACCATG AGAAGAGAGA ACAGCTTTCG TCGAACACTT AGCCGCAGTT

2851 CTAGGTCCAA TGGCAGGCAT CTGAAGTCCC TGAGAAACTC CTTGAAAACT

2901 CGGAATGTGA ATAGCCTGAA GGAAGACGAA GAACTAGTGA AAGGACAAAA

2951 ACTAATTAAG AAGGAATTCA TAGAAACTGG AAAGGTGAAG TTCTCCATCT

3001 ACCTGGAGTA CCTACAAGCA ATAGGATTGT TTTCGATATT CTTCATCATC

3051 CTTGCGTTTG TGATGAATTC TGTGGCTTTT ATTGGATCCA ACCTCTGGCT

3101 CAGTGCTTGG ACCAGTGACT CTAAAATCTT CAATAGCACC GACTATCCAG

3151 CATCTCAGAG GGACATGAGA GTTGGAGTCT ACGGAGCTCT GGGATTAGCC

3201 CAAGGTATAT TTGTGTTCAT AGCACATTTC TGGAGTGCCT TTGGTTTCGT

3251 CCATGCATCA AATATCTTGC ACAAGCAACT GCTGAACAAT ATCCTTCGAG

3301 CACCTATGAG ATTTTTTGAC ACAACACCCA CAGGCCGGAT TGTGAACAGG

3351 TTTGCCGGCG ATATTTCCAC AGTGGATGAC ACCCTGCCTC AGTCCTTGCG

3401 CAGCTGGATT ACATGCTTCC TGGGGATAAT CAGCACCCTT GTCATGATCT

3451 GCATGGCCAC tCCTGTCTTC ACcaTcATCG TcATTCCTCT tGGcAttAtt

3501 tAtGtATctG TtCAGAtGTt ttAtGtGTCt ACCTCCCGCC AGctGAGGcG

3551 TCTGGACTCT GTCACCAGgT CCCCAATcTA cTcTCAcTTC AGCGAGACCG

3601 TATCAGGTTT GCCAGTTATC cGTGCCTtTG AGCACCAGCA GcGATTTcTG

3651 AAACACAATG AGGAGAGGat TGACACCaAC CAGAAAtGTG TcTTTTccTG

3701 GATCACCTCC AACAGGtGGc TTGCAATTcG CCTGGAGcTG GTTGGGAACC

3751 TGACTGTcTT cTTTTCAGCC tTGaTGATGG TTATTTAtAG aGATACCCTA

3801 AGtGGGGACA CTGTTGGcTT TGTTcTGTCC AATGCAcTCA ATATCACACA

3851 AACCCTGAAc TGGcTGGtGa GGaTGACaTC aGaAaTaGaG aCCaACATTG

3901 tGGcTGTtGA GCGaATAACt GAGtACACAA AAGTGGAAaA TGAgGCACCc

3951 TGgGTGActG ATAAgAgGcc tCCGCCAGAT TGGcCCAGCA AAGGCAAGAT

4001 CCAGtTTAAC AAcTaCCAAg TGCGGTACCG ACCTGAgCTG GATCTGGTCC

4051 tCaGAgGGAT CACTTGTGAC ATCgGTAgCA TGGAgAAgAt TGGTGTGGTG

4101 GGCagGAcAG gAgctGGAAa gTcatcCCTC ACAAactGcC TcTTcaGaAT

4151 cTTaGaGGct GCCGGtGGTC aGATTatcAT TGATGGaGTA gAtATTGcTT

4201 cCATTgGGcT CCAcGAcCTc CGAGaGAAGc TGAcCaTcAT cCCCCAGGAc

4251 CCCATcCTGT TcTcTGGaAG cCTGAGGaTG aATcTcGACC cTTTCAACAA

4301 cTAcTCAgAT GAGGAgATTT GGAAgGCCTT GGAGcTGGcT CACcTCAAgT

4351 CTTTTGTGGC CAGcCTGCAA CTTgGGTTAT CCCACGAAGT taCAGAgGCT

4401 gGTgGCAACC TGAGCATAgG CCAgAggcag CTGCTGTGcC TGGGCAGGGc

4451 TcTGcTTCGG AAATCCAAGA TCCTGGTCCT GGATGAgGCC ACTGCTGCGG

4501 TGGATCTAGA GACAGACaAc CTCATTCAGA CGACCATCCA AAACGAgTTC

4551 GCCCACTGCA CAGTGATCAC CATCGCCCAC AGGCTGCATA CCATCATGGa

4601 CAGTGaCaAg GTaATGGtCC tAGaCaAcgG GaAgaTtAtA gAgTACGGCa

4651 gCCcTGaAGA acTgCtaCAA ATCCCTGGAc CCTTTtACtT TATGGCTAAG

4701 GaAgCTgGCA TTGAgAATgT gAaCAgCaCa aAAtTcTagc agaaggcCCC

4751 ATgGGtTaga AAagGactat AagaatAATT tCtTAtttAa ttttAttttt

4801 tataaAAtaC aGaataCata Caaaagtgtg taTaaAATGt ACgTTTTaaa

4851 aAAGGaTAag TgaacACCCa TGAACctact ACCcAgGTTA AgaaaataAa

4901 tgTCaCCAGg TacttGAgAA ACCcctcgAt TGTctACcTC gATCgTactT

4951 CCtTGcTACC caCCCctcCC AGGgacAAcC AcTgTCctGA attTcaCgAt

5001 AATtattCCt tTGCCTtTca tTTCTGTTTT ATCACCTTTG TATGTATCTT

5051 TAAACAACAT ATACCCTTTT TTACTTATGT AAATGGACTG ACTCATACTG

5101 CATACATcTT cTATGAcTTG AtTcTTTTGT tCAAtAttat AtctGagatT

5151 cAtCCATGGT GATGCAAATA GGTGCATTAt TTTTTTTCAC TGCTCTGTAg

5201 TCTGGCattg tatGaATacA gcacaatgtA tcagTtttaa Tattggggat

5251 catTagcatt atTctcaggt tttTaaaaAt tAtaagcAgt actactatgg

5301 AaaGAAAnTC TGGTCCTACA TCTCCCTGGC ACATATGTAA GAGTTTCnCn

5351 AGGGTAtAAC CtAGGAATGG AGGGTATGAA CATGTTTACA TGCACAAACT

5401 AGCtGATGCC AAACTGGTAA TGCCAACTGA AAACAtTGCT GTCAAtCTGA

5451 TGAATATGAA TTGATGTATC AATGAGAATT TCATTTGCAT TTCCCTAGTA

5501 TCTATTGGGG ATGAATATAT TTTCATGtTT CTGGGGCATT TGCATTTCCA

5551 CTTGTTTTTT aAATATTGTG TCTGATAwTT yTnnAT

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   1  TGCACTTTAA CATCTGCTTT CCCAGAGGAA AAAGTAAAGG AGAAACAGTA
  51  CAATCATAGA AGAGTCTTCG TAACAGAAGC GCGAGGAGAG CATTATGGAC
 101  AAGTTCTGCA ACTCTACTTT TTGGGATCTC TCATTACTGG AAAGTCCAGA
 151  GGCTGACCCG CCTCTTTGTT TTGAGCAAAC TGTTCTGGTG TGGATTCCCT
 201  TGGGCTTTCT TTGGCTCCTG GCTCCTTGGC AACTTTACAG CGTGTACAGA
 251  TCCAGGACCA AGAGATCTTC TATAACCAAA TTCTACCTTG CCAAGCAGGT
 301  GTTCGTCGTG TTTCTTCTTA TTTTAGCAGC CATAGACCTG TCTCTTGCGC
 351  TCACAGAAGA TACTGGACAA GCCACAGTTC CTCCTGTCAG ATATACGAAT
 401  CCAATCCTCT ACCTGTGCAC ATGGCTCCTG GTTTTGGCAG TCCAGCACAG
 451  CAGGCAATGG TGTGTACGAA AGAACTCTTG GTTCCTGTCT CTGTTCTGGA
 501  TCCTCTCGGT CTTATGCGGC GTATTCCAGT TTCAGACTCT GATACGAGCA
 551  CTCCTGAAGG ACAGCAAGTC CAACATGGCC TACTCCTACC TGTTCTTCGT
 601  CTCCTACGGT TTCCAGATTG TCCTCCTGAT TCTTACAGCC TTTTCAGGAC
 651  CAAGTGACTC AACACAAACT CCATCAGTCA CGGCTTCCTT TCTGAGTAGC
 701  ATTACATTTA GTTGGTATGA CAGGACTGTT CTGAAAGGTT ACAAGCATCC
 751  ACTGACACTA GAAGATGTCT GGGATATCGA TGAAGGGTTT AAAACAAGGT
 801  CAGTCACCAG CAAGTTTGAG GCGGCCATGA CAAAGGACCT GCAGAAAGCC
 851  AGGCAGGCTT TTCAGAGGCG GCTGCAGAAG TCCCAGCGGA AACCTGAGGC
 901  CACACTACAC GGACTGAACA AGAAGCAGAG TCAGAGCCAA GACGTTCTCG
 951  TCCTGGAAGA AGCGAAAAAG AAGTCTGAGA AGACCACCAA AGACTATCCC
1001  AAATCGTGGT TGATCAAGTC TCTCTTCAAA ACCTTCCACG TAGTGATCCT
1051  GAAATCATTT ATACTGAAAT TAATACATGA CCTTTTGGTG TTTCTGAATC
1101  CTCAGCTGCT GAAGTTGCTG ATCGGTTTCG TGAAGAGCTC TAACTCATAC
1151  GTGTGGTTTG GCTATATCTG TGCAATCCTA ATGTTTGCTG TGACTCTCAT
1201  CCAATCTTTC TGCCTTCAGT CTTACTTTCA ACATTGTTTT GTGTTGGGAA
1251  TGTGCGTACG GACAACCGTC ATGTCTTCGA TATATAAGAA GGCATTGACC
1301  CTATCTAACT TGGCTAGGAA GCAGTACACC ATTGGAGAGA CGGTGAACTT
1351  GATGTCTGTA GATTCCCAGA AGCTAATGGA TGCGACCAAC TACATGCAGT
1401  TGGTGTGGTC AAGTGTTATA CAGATTACTT TGTCCATCTT CTTCCTGTGG
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FIG. 1b

1451 AGAGAGTTGG GACCGTCCAT CTTAGCAGGT GTTGGGGTTA TGGTTCTCCT

1501 AATCCCAGTT AATGGAGTTC TGGCTACCAA GATCAGAAAT ATTCAGGTCC

1551 AAAATATGAA GAATAAAGAC AAACGTTTAA AAATCATGAA TGAGATTCTC

1601 AGTGGAATCA AGATCCTGAA ATACTTTGCC TGGGAACCTT CATTTCAAGA

1651 GCAAGTCCAG GGCATTCGGA AGAAAGAACT CAAGAACTTG CTGCGGTTCG

1701 GCCAGCTGCA GAGTCTGCTG ATCTTCATTT TACAGATAAC TCCAATCCTG

1751 GTGTCTGTGG TCACATTTTC TGTCTATGTC CTGGTGGATA GCGCCAATGT

1801 TTTGAATGCG GAGAAGGCAT TTACCTCCAT CACCCTCTTC AATATCCTAC

1851 GCTTCCCTCT GTCCATGCTT CCCATGGTGA CCTCATCGAT CCTCCAGGCC

1901 AGTGTTTCTG TGGACCGGCT GGAGAGGTAT TTGGGAGGAG ACGATTTAGA

1951 CACATCTGCC ATTCGCGCCG TCAGCAATTT TGATAAAGCT GTGAAGTTTT

2001 CAGAGGCCTC TTTTACTTGG GACCCGGACT TGGAAGCCAC AATCCAAGAT

2051 GTGAACCTGG ACATAAAGCC AGGCCAACTG GTGGCTGTGG TGGGCACTGT

2101 AGGCTCTGGG AAATCCTCTT TGGTATCAGC CATGCTGGGA GAAATGGAAA

2151 ACGTTCACGG GCACATCACC ATCCAGGGAT CCACAGCCTA TGTCCCTCAG

2201 CAGTCCTGGA TTCAGAATGG AACCATCAAA GACAACATCC TGTTTGGGTC

2251 CGAATACAAT GAAAAGAAGT ACCAGCAAGT TCTCAAAGCA TGCGCTCTCC

2301 TCCCAGACTT GGAAATATTG CCTGGAGGAG ACATGGCTGA GATCGGAGAG

2351 AAGGGGATAA ATCTCAGTGG TGGTCAGAAG CAGCGAGTCA GCCTGGCCAG

2401 AGCTGCCTAT CAAGATGCTG ACATCTATAT TCTGGACGAT CCCCTGTCGG

2451 CTGTGGATGC TCATGTGGGA AAACACATTT TCAACAAGGT TGTGGGCCCC

2501 AACGGCCTGT TGGCTGGCAA GACGAGAATC TTTGTTACTC ATGGTATTCA

2551 CTTCCTTCCC CAAGTGGATG AGATTGTAGT TCTGGGGAAA GGCACCATCT

2601 TAGAGAAAGG ATCCTATCGT GACCTGTTGG ACAAGAAGGG AGTGTTTGCT

2651 AGGAACTGGA AGACCTTCAT GAAGCATTCA GGGCCTGAAG GAGAGGCCAC

2701 AGTCAATAAT GACAGTGAGG CGGAAGACGA CGATGATGGG CTGATTCCCA

2751 CCATGGAGGA AATCCCTGAG GATGCAGCTT CCTTGGCCAT GAGAAGAGAA

2801 AATAGTCTTC GCCGTACACT GAGCCGCAGC TCTAGGTCCA GCAGCCGACG

2851 TGGGAAGTCC CTCAAAAACT CCTTGAAGAT TAAAAATGTG AATGTCTTGA

2901 AGGAGAAGGA AAAAGAAGTG GAAGGACAAA AACTAATTAA GAAAGAATTT

2951 GTGGAAACCG GGAAGGTCAA GTTCTCCATC TACCTGAAGT ATCTACAGGC

3001 AGTAGGGTGG TGGTCCATAC TTTTCATCAT CCTTTTCTAC GGATTGAATA

3051 ATGTTGCTTT TATCGGCTCT AACCTCTGGC TGAGTGCTTG GACCAGTGAC

3101 TCTGACAACT TGAATGGGAC CAACAATTCG TCTTCTCATA GGGACATGAG

3151 AATTGGGGTC TTTGGAGCTC TGGGATTAGC ACAAGGTATA TGTTTGCTTA

3201 TTTCAACTCT GTGGAGCATA TATGCTTGCA GAAATGCATC AAAAGCTTTG

3251 CACGGGCAGC TGTTAACCAA CATCCTCCGG GCACCCATGA GGTTTTTTGA

3301 CACAACTCCC ACAGGCCGGA TTGTGAACAG ATTTTCTGGT GATATTTCTA

3351 CTGTGGACGA CTTGCTCCCC CAGACACTTC GAAGCTGGAT GATGTGTTTC

3401 TTTGGCATCG CTGGCACTCT TGTCATGATC TGCATGGCCA CCCCAGTCTT

3451 CGCTATCATC ATCATTCCTC TCAGCATTCT TTATATTTCG GTGCAGGTTT

3501 TTTATGTGGC TACTTCCCGC CAGCTGAGAC GGTTGGATTC TGTCACCAAA

3551 TCTCCGATCT ATTCTCACTT CAGTGAGACT GTCACAGGTT TGCCCATTAT

3601 CCGTGCCTTT GAGCACCAGC AGCGATTTCT AGCTTGGAAT GAGAAGCAGA

3651 TTGACATCAA CCAGAAATGT GTCTTTTCCT GGATTACCTC CAACAGGTGG

3701 CTTGCAATTC GGCTGGAGCT GGTTGGAAAC TTGGTCGTCT TCTGTTCCGC

3751 CTTGCTGCTG GTTATTTATA GAAAAACCTT AACCGGGGAC GTTGTGGGCT

3801 TTGTTCTGTC CAACGCCCTC AATATCACAC AAACCTTGAA CTGGCTAGTG

3851 AGGATGACGT CAGAAGCAGA GACCAACATT GTGGCAGTTG AGCGAATAAG

3901 TGAATACATA AATGTAGAGA ATGAGGCGCC CTGGGTGACT GACAAGAGGC

3951 CTCCGGCAGA CTGGCCCAGA CATGGTGAGA TCCAGTTTAA CAACTATCAA

4001 GTGCGGTATC GGCCGGAGCT GGATCTGGTA CTGAAAGGGA TCACTTGTAA

4051 CATCAAGAGC GGAGAGAAGG TCGGCGTAGT GGGCAGGACT GGGGCTGGGA

4101 AATCATCCCT CACAAACTGC CTCTTCAGAA TCTTAGAGTC TGCGGGGGGC

4151 CAGATCATCA TTGATGGGAT AGATGTTGCC TCCATTGGAC TGCACGACCT

4201 TCGAGAGAGG CTGACCATCA TTCCCCAGGA CCCCATTTTG TTCTCGGGGA

4251 GTCTGAGGAT GAATCTCGAC CCTTTCAACA AATATTCAGA TGAGGAGGTT

4301 TGGAGGGCCC TGGAGTTGGC TCACCTCAGA TCCTTTGTGT CTGGCCTACA

4351 GCTTGGGTTG TTATCCGAAG TGACAGAGGG TGGTGACAAC CTGAGCATAG

4401 GGCAGAGGCA GCTCCTATGC CTGGGCAGGG CTGTGCTTCG AAAATCCAAA

4451 ATCCTGGTCC TGGATGAAGC CACGGCTGCA GTGGATCTGG AGACGGATAG

4501 CCTCATTCAG ACGACCATCC GAAAGGAGTT CTCCCAGTGC ACGGTCATCA

4551 CCATCGCTCA CAGGCTGCAC ACCATCATGG ACAGTGACAA GATAATGGTC

4601 CTAGACAACG GGAAGATTGT CGAGTATGGC AGTCCTGAAG AACTGCTGTC

4651 CAACAGAGGT TCCTTCTATC TGATGGCCAA GGAAGCCGGC ATTGAAAATG

4701 TGAATCACAC AGAGCTCTAG CAGCTGGTTC CGTGGCTGGC GGACTATAAG

4751 AACAGTTTCT ATTATTTGCT TTGGTTTCTG TGACTGTGCT CTAGGTGCAA

4801 AGACACATAT TTTGTTCCCG TTGCTCAGGC TGGCCTCAAA CTCTAAGGCT

4851 CCAGCAATCT CTGGTCTCAG CCAGAGACCT GTAAAAATAG ACACTTCAAA

4901 GATTATCATG AATAAATA*

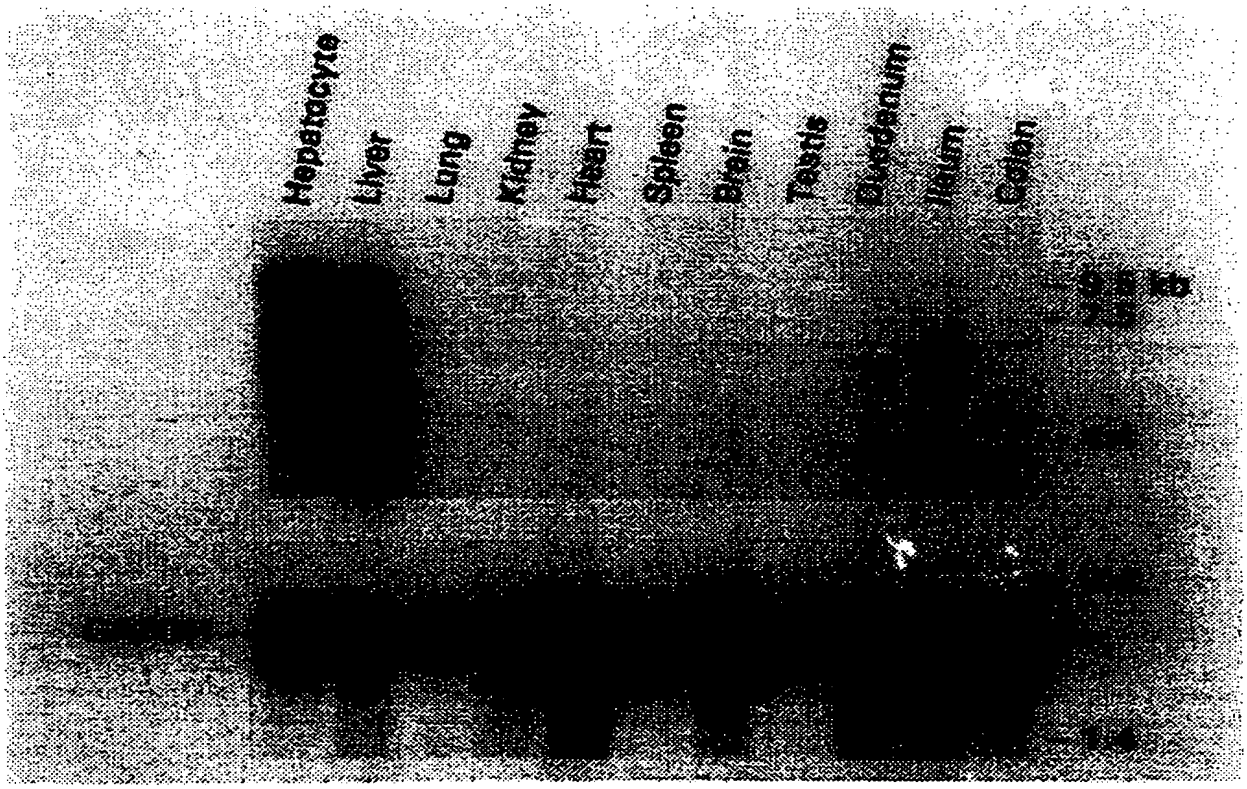

FIG. 2a

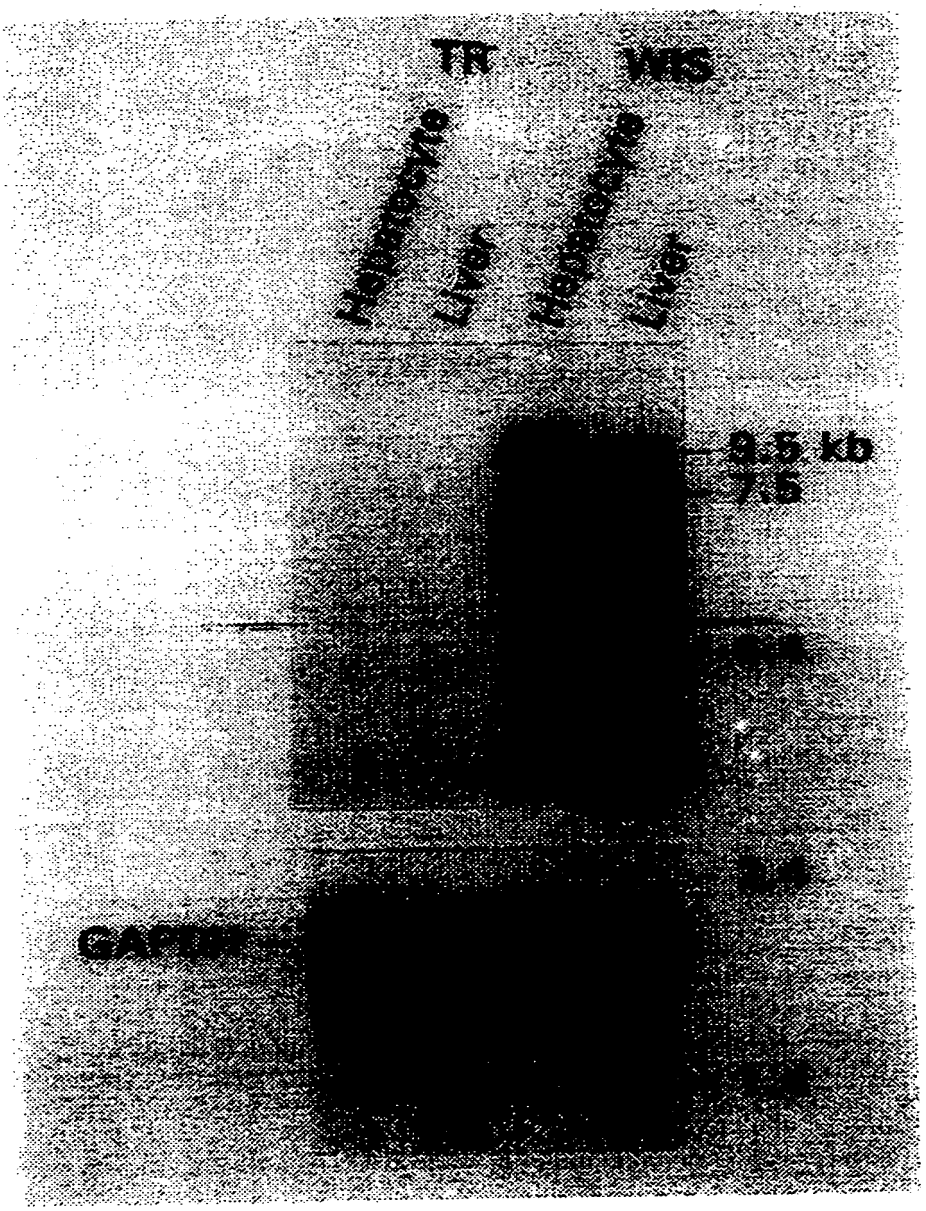

FIG. 2b

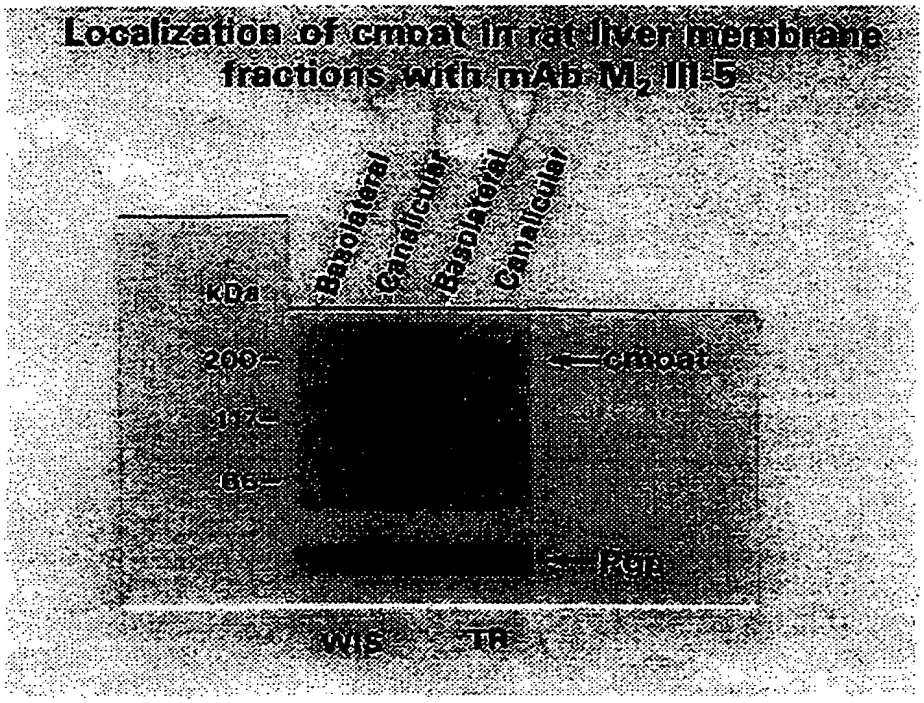

FIG. 3

```
   1 MDKFCNSTFWDLSLLESPEADPPLCFEQTVLVWIPLGFLWLLAPWQLYSVYRSRTKRSSI
  61 TKFYLAKQVFVVFLLILAAIDLSLALTEDTGQATVPPVRYTNPILYLCTWLLVLAVQHSR
 121 QWCVRKNSWFLSLFWILSVLCGVFQFQTLIRALLKDSKSNMAYSYLFFVSYGFQIVLLIL
 181 TAFSGPSDSTQTPSVTASFLSSITFSWYDRTVLKGYKHPLTLEDVWDIDEGFKTRSVTSK
 241 FEAAMTKDLQKARQAFQRRLQKSQRKPEATLHGLNKKQSQSQDVLVLEEAKKKSEKTTKD
 301 YPKSWLIKSLFKTFHVVILKSFILKLIHDLLVFLNPQLLKLLIGFVKSSNSYVWFGYICA
                                          *
 361 ILMFAVTLIQSFCLQSYFQHCFVLGMCVRTTVMSSIYKKALTLSNLARKQYTIGETVNLM
 421 SVDSQKLMDATNYMQLVWSSVIQITLSIFFLWRELGPSILAGVGVMVLLIPVNGVLATKI
 481 RNIQVQNMKNKDKRLKIMNEILSGIKILKYFAWEPSFQEQVQGIRKKELKNLLRFGQLQS
 541 LLIFILQITPILVSVVTFSVYVLVDSANVLNAEKAFTSITLFNILRFPLSMLPMVTSSIL
 601 QASVSVDRLERYLGGDDLDTSAIRAVSNFDKAVKFSEASFTWDPDLEATIQDVNLDIKPG
 661 QLVAVVGTVGSGKSSLVSAMLGEMENVHGHITIQGSTAYVPQQSWIQNGTIKDNILFGSE
                                    ||:|:.||.|||||||.||||:.:::||||||..
                                rMRP1  GHVTLKGSVAYVPQQAWIQNDSLRENILFGRP
 721 YNEKKYQQVLKACALLPDLEILPGGDMAEIGEKGINLSGGQKQRVSLARAAYQDADIYIL
     ..|. |..|:.||||||||||||||:||:.|||||:   |
     LQEHCYKAVMEACALLPDLEILPSGDLTEIGEKGVITS  rMRP1
 781 DDPLSAVDAHVGKHIFNKVVGPNGLLAGKTRIFVTHGIHFLPQVDEIVVLGKGTILEKGS
 841 YRDLLDKKGVFARNWKTFMKHSGPEGEATVNNDSEAEDDDDGLIPTMEEIPEDAASLAMR
 901 RENSLRRTLSRSSRSSSRRGKSLKNSLKIKNVNVLKEKEKEVEGQKLIKKEFVETGKVKF
 961 SIYLKYLQAVGWWSILFIILFYGLNNVAFIGSNLWLSAWTSDSDNLNGTNNSSSHRDMRI
1021 GVFGALGLAQGICLLISTLWSIYACRNASKALHGQLLTNILRAPMRFFDTTPTGRIVNRF
1081 SGDISTVDDLLPQTLRSWMMCFFGIAGTLVMICMATPVFAIIIPLSILYISVQVFYVAT
1141 SRQLRRLDSVTKSPIYSHFSETVTGLPIIRAFEHQQRFLAWNEKQIDINGKCVFSWITSN
1201 RWLAIRLELVGNLVVFCSALLLVIYRKTLTGDVVGFVLSNALNITQTLNWLVRMTSEAET
1261 NIVAVERISEYINVENEAPWVTDKRPPADWPRHGEIQFNNYQVRYRPELDLVLKGITCNI
1321 KSGEKVGVVGRTGAGKSSLTNCLFRILESAGGQIIIDGIDVASIGLHDLRERLTIIPQDP
1381 ILFSGSLRMNLDPFNKYSDEEVWRALELAHLRSFVSGLQLGLLSEVTEGGDNLSIGQRQL
1441 LCLGRAVLRKSKILVLDEATAAVDLETDSLIQTTIRKEFSQCTVITIAHRLHTIMDSDKI
1501 MVLDNGKIVEYGSPEELLSNRGSFYLMAKEAGIENVNHTEL
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FIG. 4

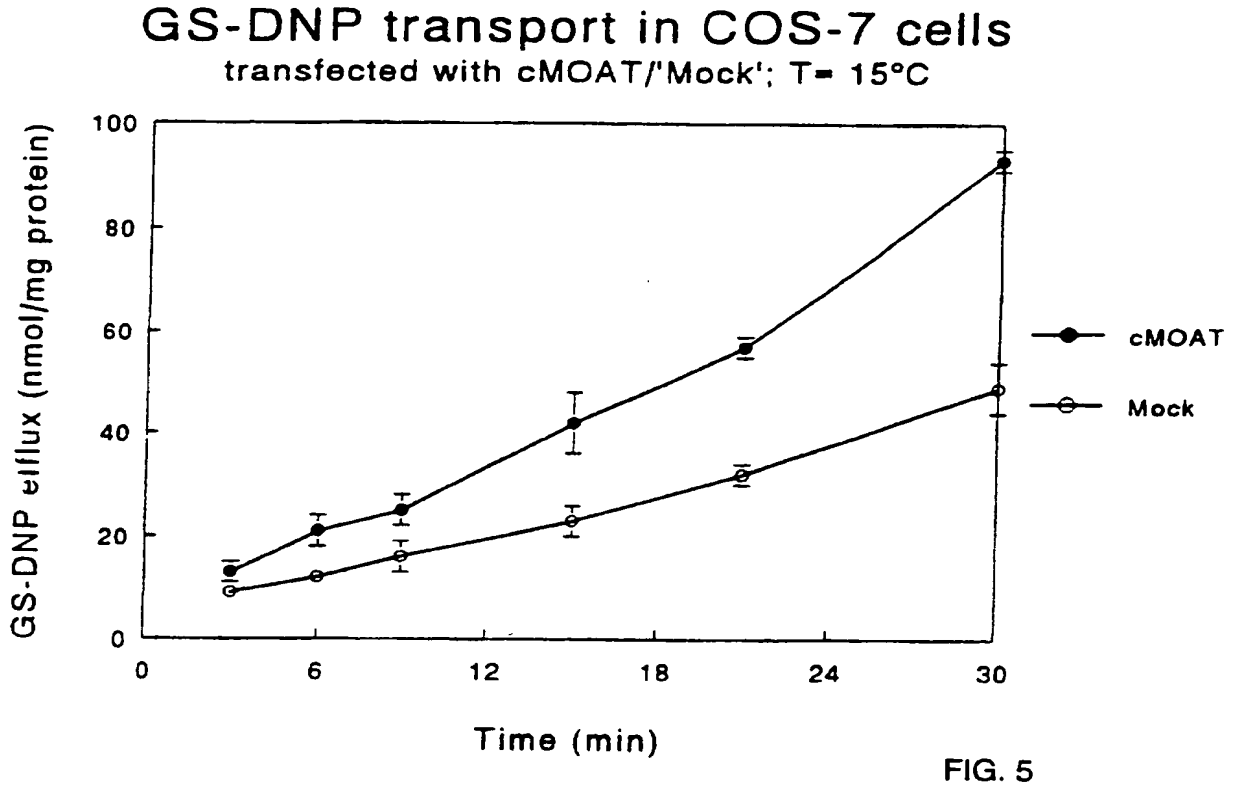

FIG. 5

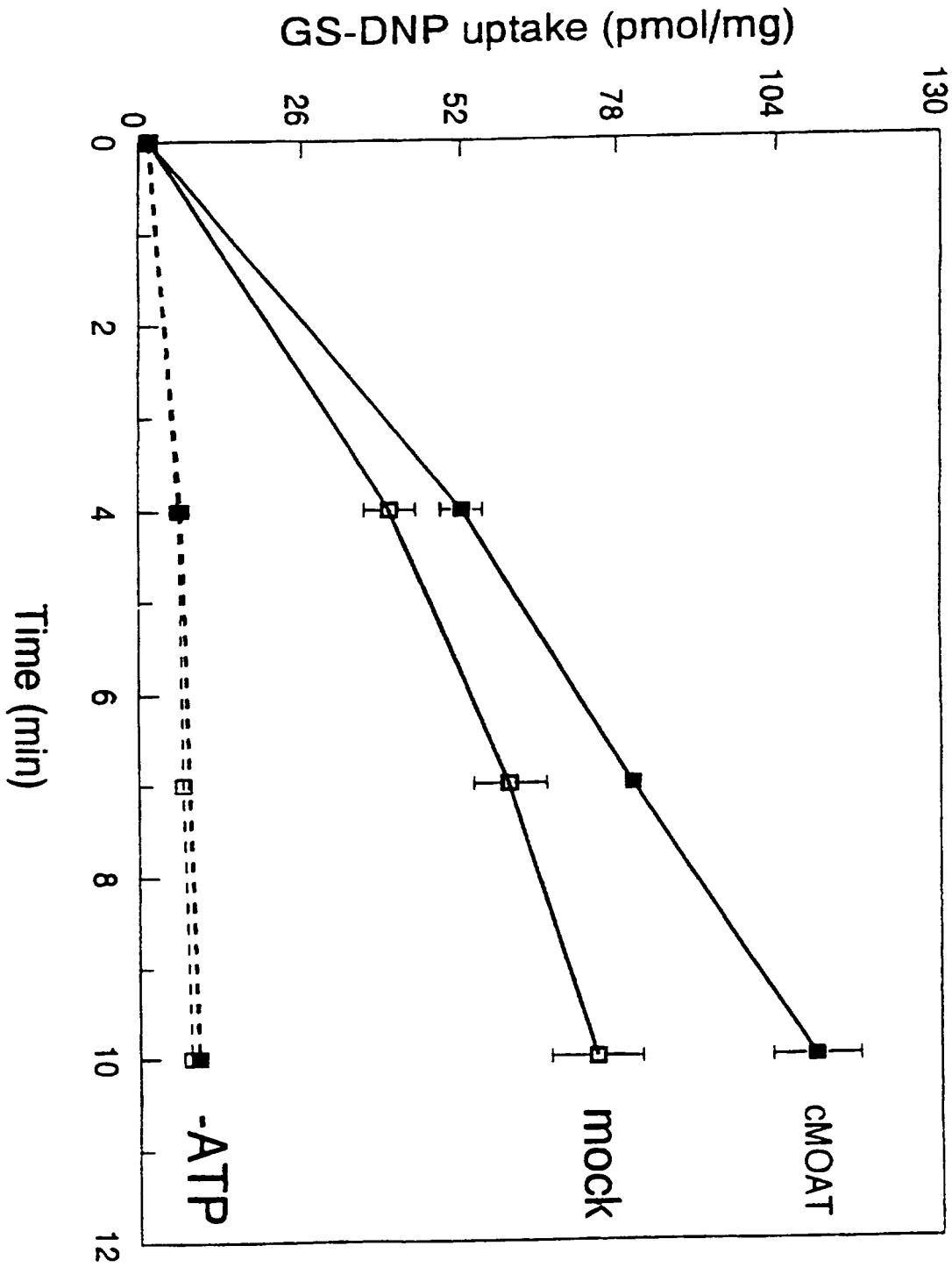
GS-DNP uptake (pmol/mg)
0
26
52
78
104
130
Time (min)
0
2
4
6
8
10
12
-ATP
mock
cMOAT

FIG. 6

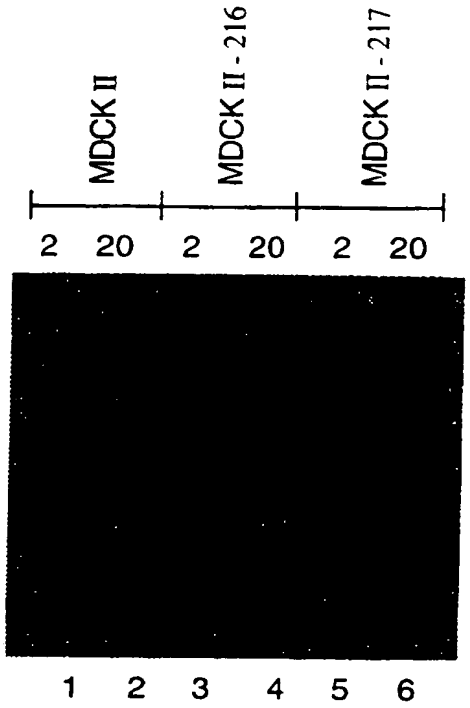

FIG. 7

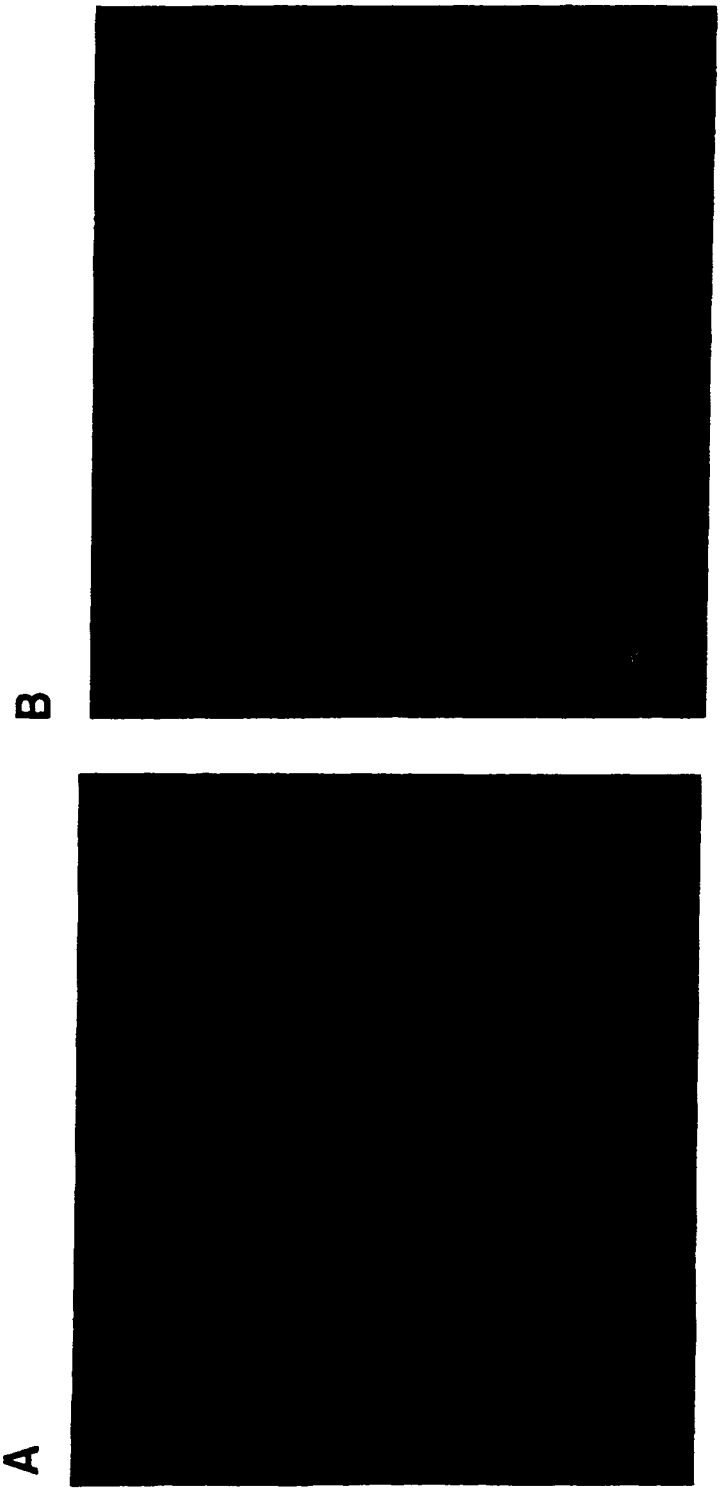

FIG. 8

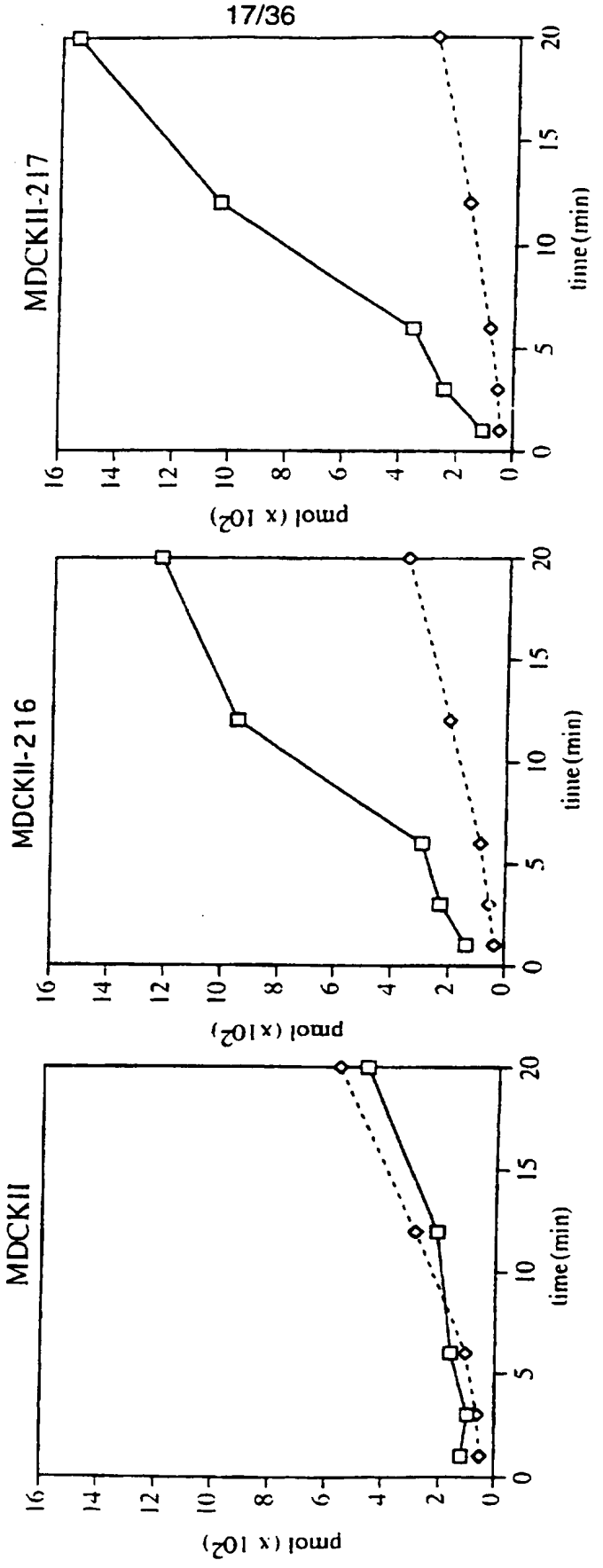

FIG. 9

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   1 MLEKFCNSTF WNSSFLDSPE ADLPLCFEQT VLVWIPLGFL VLLAPWQLLH VYKSRTKRSS TTKLYLAKQV FVGFLLILAA IELALVLTED SGQATVPAVR
 101 YTNPSLYLGT WLLVLLIQYS RQWCVQKNSW FLSLFWILSI LCGTFQFQTL IRTLLQGDNS NLAYSCLFFI SYGFQILILI FSAFSENNES SNWPSSIASF
 201 LSSITYSWYD SIILKGYKRP LTLEDVWEVD EEMKTKTLVS KFETHMKREL QKARRALQRR QEKSSQQNSG ARLPGLNKNQ SQSQDALVLE DVEKKKKKSG
 301 TKKDVPKSWL MKALFKTFYM VLLKSFLLKL VNDIFTFVSP QLLKLLISFA SDRDTYLWIG YLCAILLFTA ALIQSFCLQC YFQLCFKLGV KVRTAIMASV
 401 YKKALTLSNL ARKEYTVGET VNLMSVDAQK LMDVTNFMHM LWSSVLQIVL SIFFLWRELG PSVLAGVGVM VLVIPINAIL STKSKTIQVK NMKNKDKRLK
 501 IMNEILSGIK ILKYFAWEPS FRDQVQNLRK KELKNLLAFS QLQCVVIFVF QLTPVLVSVV TFSVYVLVDS NNILDAQKAF TSITLFNILR FPLSMLPMMI
 601 SSMLQASVST ERLEKYLGGD DLDTSAIRHD CNFDKAMQFS EASFTWEHDS EATVRDVNLD IMAGQLVAVI GPVGSGKSSL ISAMLGEMEN VHGHITIKGT
 701 TAYVPQQSWI QNGTIKDNIL FGTEFNEKRY QQVLEACALL PDLEMLPGGD LAEIGEKGIN LSGGQKQRIS LARATYQNLD IYLLDDPLSA VDAHVGKHIF
 801 NKVLGPNGLL KGKTRLLVTH SMHFLPQVDE IVVLGNGTIV EKGSYSALLA KKGEFAKNLK TFLRHTGPEE EATVHDGSEE EDDDYGLISS VEEIPEDAAS
 901 ITMRRENSFR RTLSRSSRSN GRHLKSLRNS LKTRNVNSLK EDEELVKGQK LIKKEFIETG KVKFSIYLEY LQAIGLFSIF FIILAFVMNS VAFIGSNLWL
1001 SAWTSDSKIF NSTDYPASQR DMRVGVYGAL GLAQGIFVFI AMFWSAFGFV HASNILHKQL LNNILRAPMR FFDTTPTGRI VNRFAGDIST VDDTLPQSLR
1101 SWITCFLGII STLVMICMAT PVFTIIVIPL GIIYVSVQMF YVSTSRQLRR LDSVTRSPIY SHFSETVSGL PVIRAFEHQQ RFLKHNEERI DTNQKCVFSW
1201 ITSNRWLAIR LELVGNLTVF FSALMMVIYR DTLSGDTVGF VLSNALNITQ TLNWLVRMTS EIETNIVAVE RITEYTKVEN EAPWVTDKRP PPDWPSKGKI
1301 QFNNYQVRYR PELDLVLRGI TCDIGSMEKI GVVGRTGAGK SSLTNCLFRI LEAAGGQIII DGVDIASIGL HDLREKLTII PQDPILFSGS LRMNLDPFNN
1401 YSDEEIWKAL ELAHLKSFVA SLQLGLSHEV TEAGGNLSIG QRQLLCLGRA LLRKSKILVL DEATAAVDLE TDNLIQTTIQ NEFAHCTVIT IAHRLHTIMD
1501 SDKVMVLDNG KIIEYGSPEE LLQIPGPFYF MAKEAGIENV NSTKF*
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FIG. 10

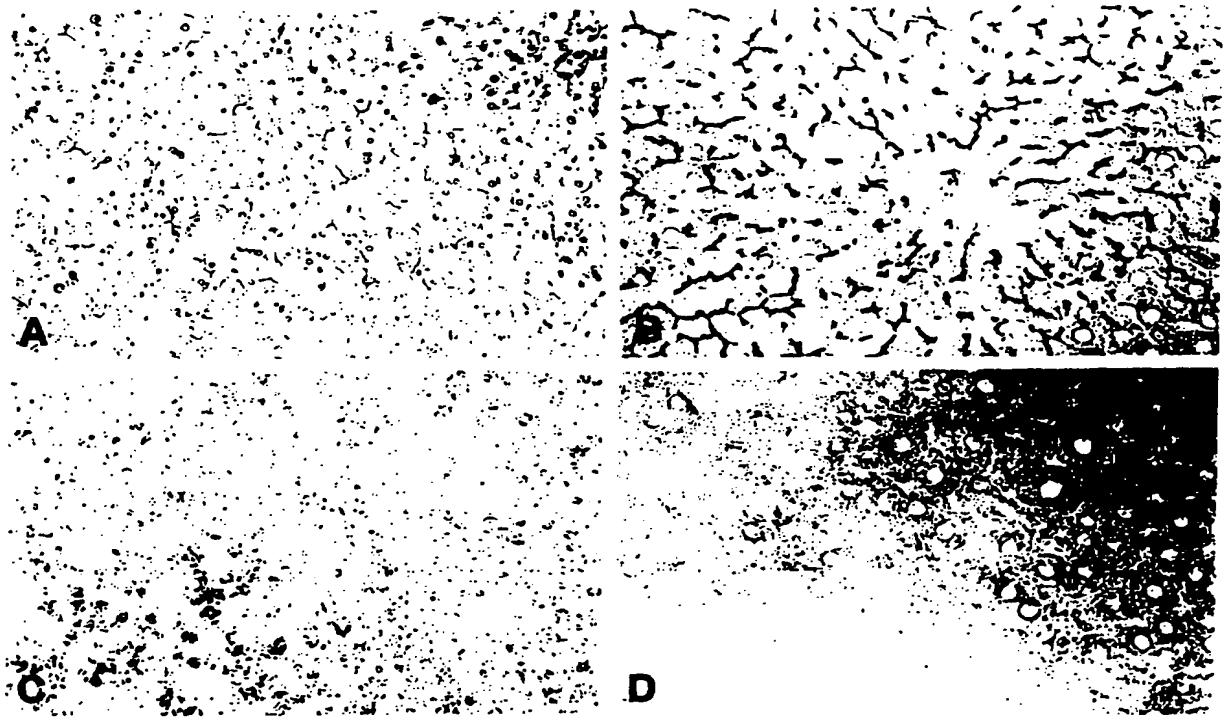


FIG. 11

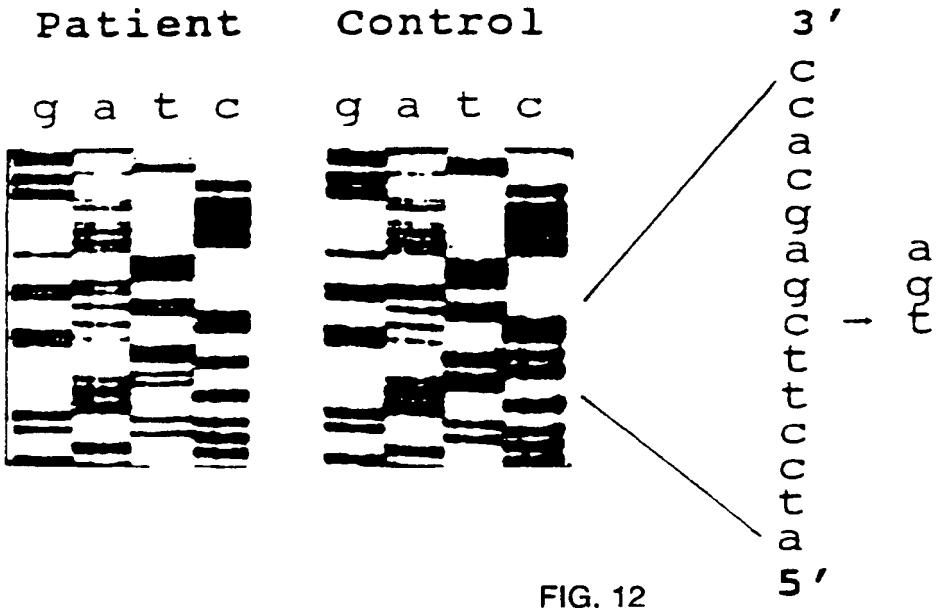


FIG. 12

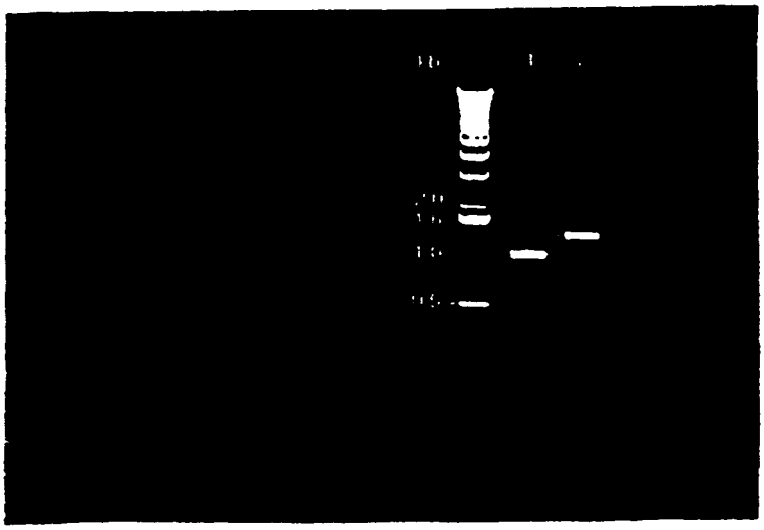


FIG. 13

```
                                    ** * ** *** ****          *  *     * * ***
cMOAT/  MRP1  FVLRHINVTI NGGEKVGIVG RTGAGKSSLT LGLFRINESA EGEIIIDGIN
        MRP2  LVLRGITCDI GSMEKIGVVG RTGAGKSSLT NCLFRILEAA GGQIIIDGVD
        MRP3  LVLRDLSLHV HGGEKVGIVG RTGAGKSSMT LACSRILEAA KGEIRIDGLN
        MRP4  .......... .......... .......... .......... ..........
        MRP5  .......TI KPKEKIGIVG RTGSGKSSLG MALFRLVELS GGCIKIDGVR
        HSUR  PVLKHVNALI SPGQKIGICG RTGSGKSSFS LAFFRMVDTF EGHIIIDGID

                 ** * *    ***  *  ****  * * ****  *      *  ** *
cMOAT/  MRP1  IAKIGLHDLR FKITIIPQDP VLFSGSLRMN LDPFSQYSDE EVWTSLELAH
        MRP2  IASIGLHDLR EKLTIIPQDP ILFSGSLRMN LDPFNNYSDE EIWKALELAH
        MRP3  VADIGFHDVR CQMTIIPRDP ILFSGTLRMN LDPFGSYSEE DIWWALELSH
        MRP4  .......... .......... .......... .......... ..........
        MRP5  ISDIGLADLR SKLSIIPQEP VLFSGTVRSN LDPFNQYTED QIWDALERTH
        HSUR  IAKLPLHTLR SRLSIILQDP VLFSGTIRFN LDPERKCSDS TLWEALEIAQ

                 *    *  *  * *  * *******  *********    ** *****
cMOAT/  MRP1  LKDFVSALPD KLDHECAEGG ENLSVGQRQL VCLARALLRK TKILVLDEAT
        MRP2  LKSFVASLQL GLSHEVTEAG GNLSIGQRQL LCLGRALLRK SKILVLDEAT
        MRP3  LHTFVSSQPA GLDFQCSEGG ENLSVGQRQL VCLARALLRK SRILVLDEAT
        MRP4  LKETIEDLPG KMDTELAESG SNFSVGQRQL VCLARAILRK NQILIIDEAT
        MRP5  MKECIAQLPL KLESEVMENG DNFSVGERQL LCIARALLRH CKILILDEAT
        HSUR  LKLVVKALPG GLDAIITEGG ENFSQGQRQL FCLARAFVRK TSIFIMDEAT

                 ** * *** * ** *** *  *********  **** *     *** *   *
cMOAT/  MRP1  AAVDLETDDL IQSTIRTQFE DCTVLTIAHR LNTIMDYTRV IVLDKGEIQE
        MRP2  AAVDLETDNL IQTTIQNEFA HCTVITIAHR LHTIMDSDKV MVLDNGKIIE
        MRP3  AAIDLETDNL IQATIRTQFD TCTVLTIAHR LNTIMDYTRV LVLDKGVVAE
        MRP4  ANVDPRTDEL IQKKIREKFA HCTVLTIAHR LNTIIDSDKI MVLDSGRLKE
        MRP5  AAMDTETDLL IQETIREAFA DCTMLTIAHR LHTVLGSDRI MVLAQGQVVE
        HSUR  ASIDMATENI LQKVVMTAFA DRTVVTIAHR VHTILSADLV IVLKRGAILE

                    *  **     **  *    **
cMOAT/  MRP1  YGAPSDLLQQ R.GLFYSMAK DAGL
        MRP2  YGSPEELLQI P.GPFYFMAK EAGI
        MRP3  FDSPANLIAA R.GIFYGMAR DAGL
        MRP4  YDEPYVLLQN KESLFYKMVQ QLG.
        MRP5  FDTPSVLLSN DSSRFYAMFA AAE.
        HSUR  FDKPEKLLSR KDSVFASFVR ADK.
```

FIG. 14

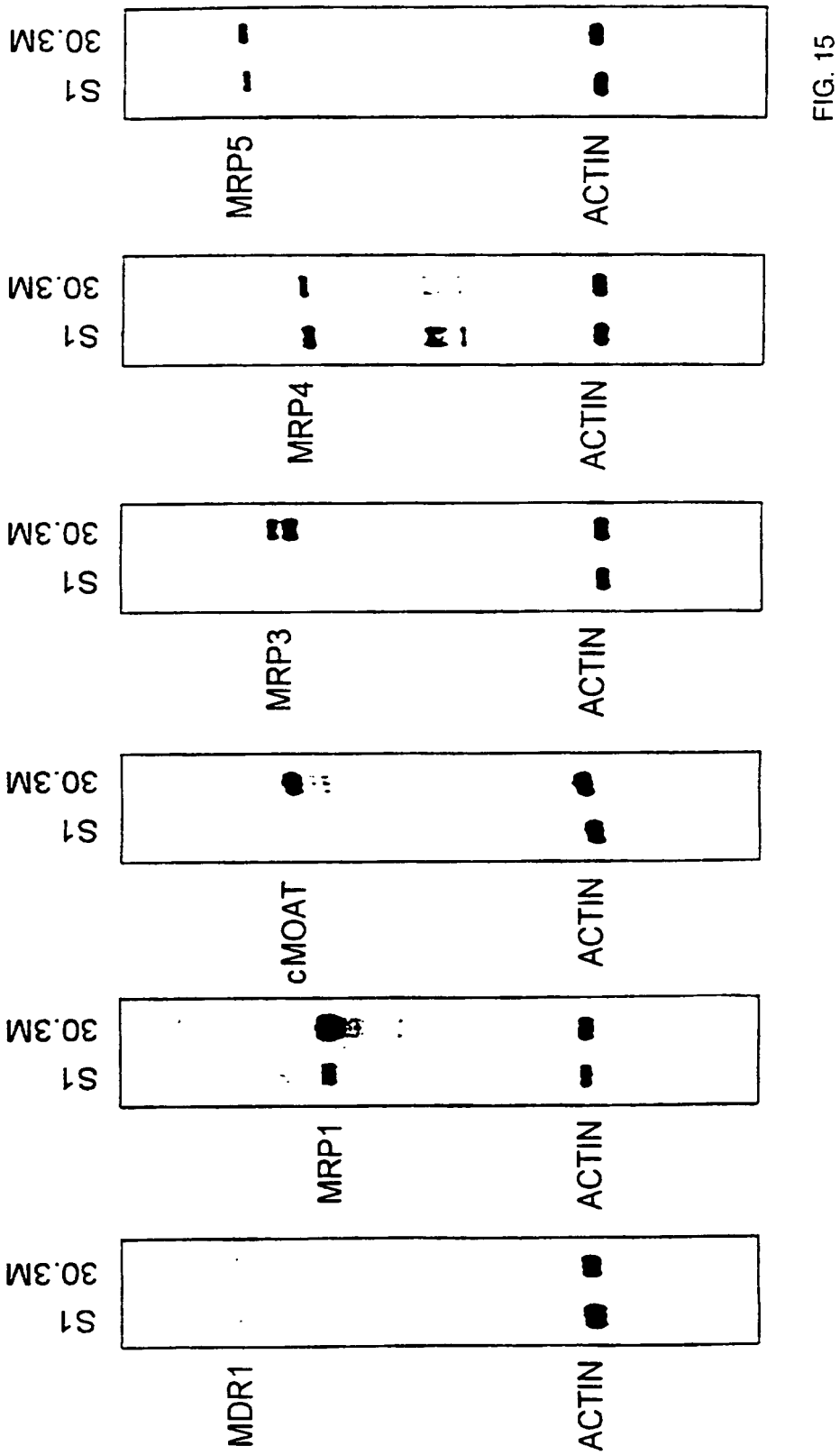
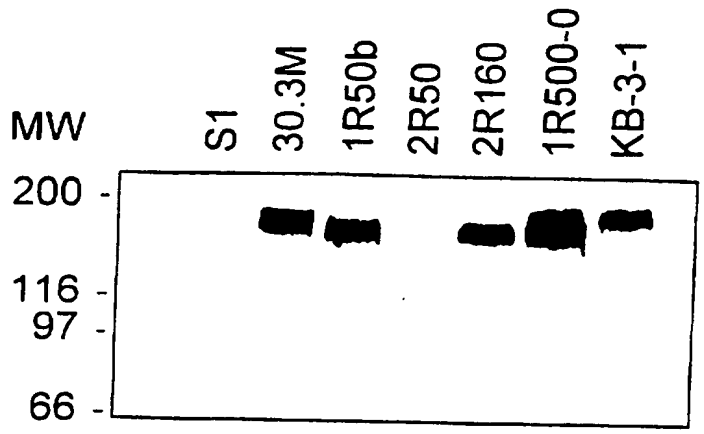

S1
30.3M
MRP5
ACTIN
S1
30.3M
MRP4
ACTIN
S1
30.3M
MRP3
ACTIN
S1
30.3M
cMOAT
ACTIN
S1
30.3M
MRP1
ACTIN
S1
30.3M
MDR1
ACTIN

FIG. 15



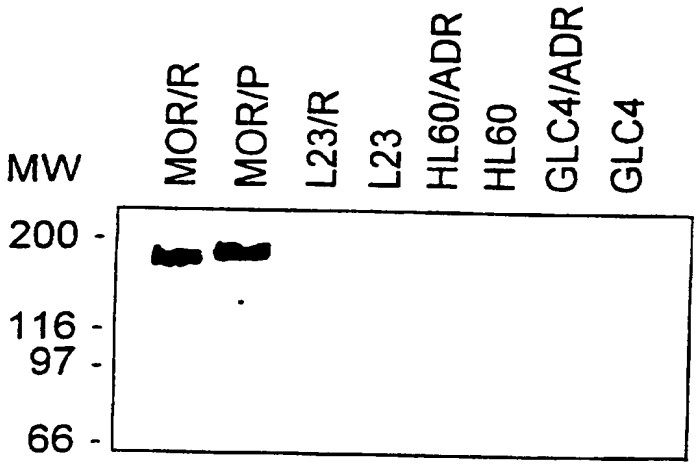
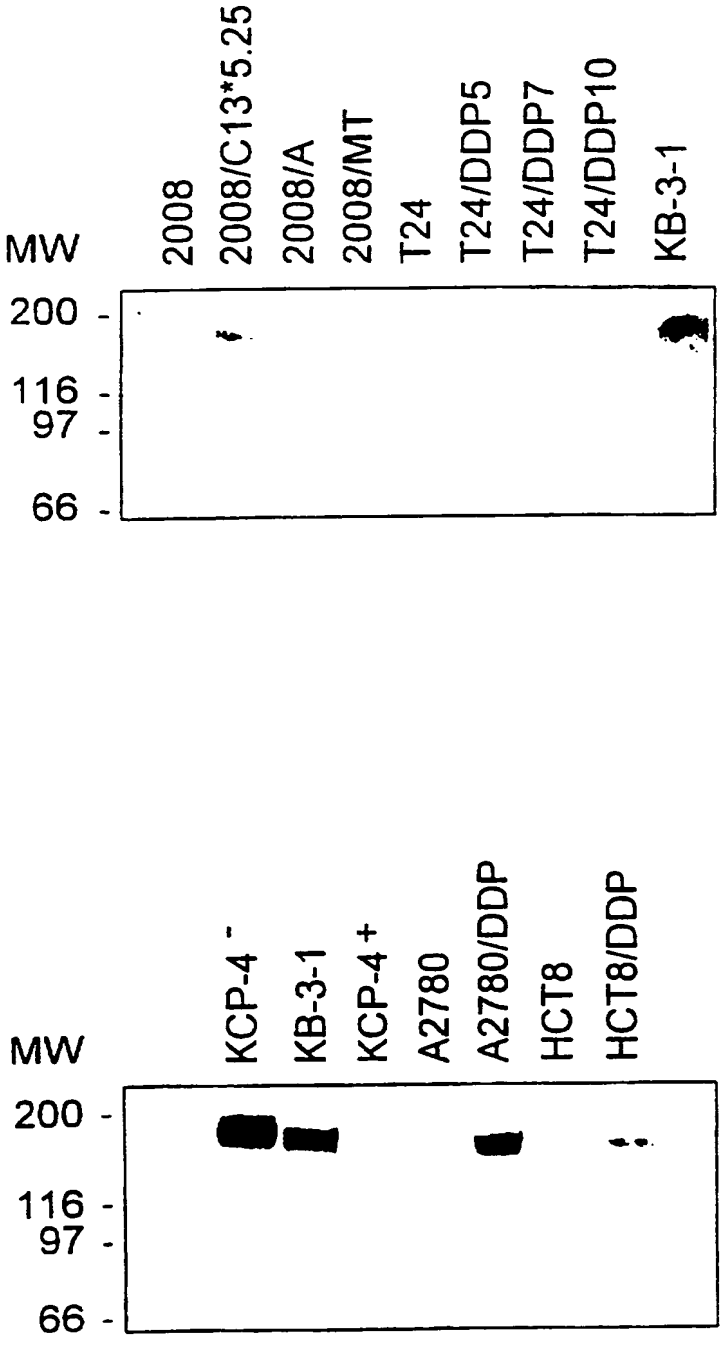


FIG. 16a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2008
2008/C13*5.25
2008/A
2008/MT
T24
T24/DDP5
T24/DDP7
T24/DDP10
KB-3-1
200
116
97
66
MW
KCP-4 -
KB-3-1
KCP-4 +
A2780
A2780/DDP
HCT8
HCT8/DDP
200
116
97
66

FIG. 16b

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  51  TGCAGCcGCG CcTCGGCCCC ATGGACGCCC TGTGCGGTTC CGGGGAGcTC
 101  GGCTCCAAgt tCTGGGACTC CAACCTGTCT GTGCACACAG AAAACCCGGA
 151  CCTCACTCCC TGCTTCCAGA ACTCCCTGCT GGCCTGGGTG CCCTGCATcT
 201  ACCTGTGGGT CGCCCTGCCC TGCTACTTGC TcTACcTGCG GCACCATTGT
 251  cGTGGCTACA TCATCCTCTC CCACCTGTCC AAGCTCAAGA TGGTCCTGGG
 301  TGTCCTGCTG TGnnnngtgt gctctcctgg cgacctttt tactcttcca
 351  tggcctggtc catggccgcc cctgcccctg ttttctttgt cacccccttg
 401  gtggtggggg tcaccatgCT GCTGGCCACC CTGCTGATAC AGTATGAsCG
 451  GCTGCAGGGC GTACAGTCTT CGGGGGTCCT CATTATCTTC TGGTTCCTGT
 501  GTGTGGTCTG CGCCATCGTC CCATTCCGCT CCAAGATCCT TTTAGCCAAG
 551  GCAGAGGGTG AGATCTCAGA CCCCTTCCGC TTCACCACCT TCTACATCCA
 601  CTTTGCCCTG GTACTCTCTG CCCTCATCTT GGCCTGCTTC AGGGAGAAAC
 651  CTCCATTTTT CTCCGCAAAG AATGTCGACC CTAACCCCTA CCCTGAGACC
 701  AGCGCTGGCT TTCTCTCCCG CCTGTTTTTC TGGTGGTTCA CAAAGATGGC
 751  CATCTATGGC TACCGGCATC CCCTGGAGGA GAAGGACCTC TGGTCCCTAA
 801  AGGAAGAGGA CAGATCCCAG ATGGTGGTGC AGCAGCTGCT GGAGGCATGG
 851  AGGAAGCAGG AAAAGCAGAC GGCACGACAC AAGGCTTCAG CAGCACCTGG
 901  GAAAAATGCC TCCGGCGAGG ACGAGGTGCT GCTGGGTGCC CGGCCCAGGC
 951  CCCGGAAGCC CTCCTTCCTG AAGGCCCTGC TGGCCACCTT CGGCTCCAGC
1001  TTCCTCATCA GTGCCTGCTT CAAGCTTATC CAGGACCTGC TCTCCTTCAT
1051  CAATCCACAG CTGCTCAGCA TCCTGATCAG GTTTATCTCC AACCCCATGG
1101  CCCCCTCCTG GTGGGGCTTC CTGGTGGCTG GGCTTGATGT TCCTGTTGCT
1151  CCATGGATGC AGTCGCTGAT CTTACAACAC TATTACCACT ACATCTTTGT
1201  GACTGGGGTG AAGTTTCGTA CTGGGATCAT GGGTGTCATC TACAGGAAGG
1251  CTCTGGTTAT CACCAACTCA GTCAAACGTG CGTCCACTGT GGGGGAAATT
```

FIG. 17a

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1351 CCTCAATCTG CTGTGGTCAG CACCCCTGCA GATCATCCTG GCGATCTACT

1401 TCCTCTGGCA GAACCTAGGT CCCTCTGTCC TGGCTGGAGT CGCTTTCATG

1451 GTCTTGCTGA TTCCACTCAA CGGAGCTGTG GCCGTGAAGA TGCGCGCCTT

1501 CCAGGTAAAG CAAATGAAAT TGAAGGACTC GCGCATCAAG CTGATGAGTG

1551 AGATCCTGAA CGGCATCAAG GTGCTGAAGC TGTACGCCTG GGAGCCCAGC

1601 TTCCTGAAGC AGGTGGAGGG CATCAGGCAG GGTGAGCTCC AGCTGCTGCG

1651 CACGGCGGCC TACCTCCACA CCACAACCAC CTTCACCTGG ATGTGCAGCC

1701 CCTTCCTGGT GACCCTGATC ACCCTCTGGG TGTACGTGTA CGTGGACCCA

1751 AACAATGTGC TGGACGCCGA GAAGGCCTTT GTGTCTGTGT CCTTGTTTAA

1801 TATCTTAAGA CTTCCCCTCA ACATGCTGCC CCAGTTAATC AGCAACCTGA

1851 CTCAGGCCAG TGTGTCTCTG AAACGGATCC AGCAATTCCT GAGCCAAGAG

1901 GAACTTGACC CCCAGAGTGT GGAAAGAAAG ACCATCTCCC CAGGCTATGC

1951 CATCACCATA CACAGTGGCA CCTTCACCTG GGCCCAGGAC CTGCCCCCCA

2001 CTCTGCACAG CCTAGACATC CAGGTCCCGA AAGGGGCACT GGTGGCCGTG

2051 GTGGGGCCTG TGGGCTGTGG GAAGTCCTCC CTGGTGTCTG CCCTGCTGGG

2101 AGAGATGGAG AAGCTAGAAG GCAAAGTGCA CATGAAGGGC TCCGTGGCCT

2151 ATGTGCCCCA GCAGGCATGG ATCCAGAACT GCACTCTTCA GGAAAACGTG

2201 CTTTTCGGCA AAGCCCTGAA CCCCAAGCGC TAcCAGCAGA CTCTGGAGGC

2251 CTGTGCCTTG CTAGCTGACC TGGAGATGCT GCCTGGTGGG GATCAGACAG

2301 aGATTGGAGA gAAGGGCATT AACCTGTCTG GGGGCCAGCG GCAGCGbGTC

2351 AGTCTGGCTC GAGCTGTTTA CAGTGATGCC GaTATTTTCT TGCTGGATGA

2401 CCCACTGTCC GCGGTGGACT CTCATGTGGC CAAGCACATC TTTGACCACG

2451 TCATCGGGCC AGAAGGCGTG CTGGCAGGCA AGACGCGAGT GCTGGTGACG

2501 CACGGCATTA GCTTCCTGCC CCAGACAGAC TTCATCATTG TGCTAGCTGA

2551 TGGACAGGTG TCTGAGATGG GCCCGTACCC AGCCCTGCTG CAGCGCAACG

2601 GCTCCTTTGC CAACTTTCTC TGCAACTATG CCCCCGATGA GGACCAAGGG

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2701 ACTGCTGATT GAAGACACAC TCAGCAACCA CACGGATCTG ACAGACAATG

2751 ATCCAGTCAC CTATGTGGTC CAGAAGCAGT TTATGAGACA GCTGAGTGCC

2801 CTGTCCTCAG ATGGGGAGGG ACAGGGTCGG CCTGTACCCC GGAGGCACCT

2851 GGGTCCATCA GAGAAGGTGC AGGTGACAGA GGCGAAGGCA GATGGGGCAC

2901 TGACCCAGGA GGAGAAAGCA GCCATTGGCA CTGTGGAGCT CAGTGTGTTC

2951 TGGGATTATG CCAAGGCCGT GGGGCTCTGT ACCACGCTGG CCATCTGTCT

3001 CCTGTATGTG GGTCAAAGTG CGGCTGCCAT TGGAGCCAAT GTGTGGCTCA

3051 GTGCCTGGAC AAATGATGCC ATGGCAGACA GTAGACAGAA CAACACTTCC

3101 CTGAGGCTGG GCGTCTATGC TGCTTTAGGA ATTCTGCAAG GGTTCTTGGT

3151 GATGCTGGCA GCCATGGCCA TGGCAGCGGG TGGCATCCAG GCTGCCCGTG

3201 TGTTGCACCA GGCACTGCTG CACAACAAGA TACGCTCGCC ACAGTCCTTC

3251 TTTGACACCA CACCATCAGG CCGCATCCTG AACTGCTTCT CCAAGGACAT

3301 CTATGTCGTT GATGAGGTTC TGGCCCCTGT CATCCTCATG CTGCTCAATT

3351 CCTTCTTCAA CGCCATCTCC ACTCTTGTGG TCATCATGGC CAGCACGCCG

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3551 GTCATCCGGG CCTACAACCG CAGCCGGGAT TTTGAGATCA TCAGTGATAC

3601 TAAGGTGGAT GCCAACCAGA GAAGCTGCTA CCCCTACATC ATCTCCAACC

3651 GGTGGCTGAG CATCGGAGTG GAGTTCGTGG GGAACTGCGT GGTGCTCTTT

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4201 cTCGGGGACC CTGCGCATGA ACCTGGAcCC CTTCGGCAGC TACTCAGAGG

4251 AGGACATTTG GTGGGCTTTG GAGCTGTCCC ACCTGCACAC GTTTGTgAGC

4301 TCCCAGCCGG CAGGCCTGGA CTTCCAGTGC TCAGAGGGCG GGGAGAATCT

4351 CAGCGTGGGC CAGAGGCAGC TCGTGTGCCT GGCCCGAGCC CTGCTCCGCA

4401 AGAGCCGCAT CCTGGTTTTA GACGAGGCCA CAGCTGCCAT CGACCTGGAG

4451 ACTGACAACC TCATCCAGGC TACCATCCGC ACCCAGTTTG ATACCTGCAC

4501 TGTCCTGACC ATCGCACACC GGCTTAACAC TATCATGGAC TACACCAGGG

4551 TCCTGGTCCT GGACAAAGGA GTAGTAGCTG AGTTTGATTC TCCAGCCAAC

4601 CTCATTGCAG CTAGAGGCAT CTTCTACGGG ATGGCCAGAG ATGCTGGACT

4651 TGCCTAAAAT ATATTCCTGA GATTTCCTCC TGGCCTTTCC TGGTTTTCAT

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 301  KALLATFGSS FLISACFKLI QDLLSFINPQ LLSILIRFIS NPMAPSWWGF
 351  LVAGLDVPVA PWMQSLILQH YYHYIFVTGV KFRTGIMGVI YRKALVITNS
 401  VKRASTVGEI VNLMSVDAQR FMDLAPFLNL LWSAPLQIIL AIYFLWQNLG
 451  PSVLAGVAFM VLLIPLNGAV AVKMRAFQVK QMKLKDSRIK LMSEILNGIK
 501  VLKLYAWEPS FLKQVEGIRQ GELQLLRTAA YLHTTTTFTW MCSPFLVTLI
 551  TLWVYVYVDP NNVLDAEKAF VSVSLFNILR LPLNMLPQLI SNLTQASVSL
 601  KRIQQFLSQE ELDPQSVERK TISPGYAITI HSGTFTWAQD LPPTLHSLDI
 651  QVPKGALVAV VGPVGCGKSS LVSALLGEME KLEGKVHMKG SVAYVPQQAW
 701  IQNCTLQENV LFGKALNPKR YQQTLEACAL LADLEMLPGG DQTEIGEKGI
 751  NLSGGQRQRV SLARAVYSDA DIFLLDDPLS AVDSHVAKHI FDHVIGPEGV
 801  LAGKTRVLVT HGISFLPQTD FIIVLADGQV SEMGPYPALL QRNGSFANFL
 851  CNYAPDEDQG HLEDSWTALE GAEDKEALLI EDTLSNHTDL TDNDPVTYVV
 901  QKQFMRQLSA LSSDGEGQGR PVPRRHLGPS EKVQVTEAKA DGALTQEEKA
 951  AIGTVELSVF WDYAKAVGLC TTLAICLLYV GQSAAAIGAN VWLSAWTNDA
1001  MADSRQNNTS LRLGVYAALG ILQGFLVMLA AMAMAAGGIQ AARVLHQALL
1051  HNKIRSPQSF FDTTPSGRIL NCFSKDIYVV DEVLAPVILM LLNSFFNAIS
1101  TLVVIMASTP LFTVVILPLA VLYTLVQRFY AATSRQLKRL ESVSRSPIYS
1151  HFSETVTGAS VIRAYNRSRD FEIISDTKVD ANQRSCYPYI ISNRWLSIGV
1201  EFVGNCVVLF AALFAVIGRS SLNPGLVGLS VSYSLQVTFA LNWMIRMMSD
1251  LESNIVAVER VKEYSKTETE APWVVEGSRP PEGWPPRGEV EFRNYSVRYR
```

FIG. 17b

1301 PGLDLVLRDL SLHVHGGEKV GIVGRTGAGK SSMTLCLFRI LEAAKGEIRI

1351 DGLNVADIGL HDLRSQLTII PQDPILFSGT LRMNLDPFGS YSEEDIWWAL

1401 ELSHLHTFVS SQPAGLDFQC SEGGENLSVG QRQLVCLARA LLRKSRILVL

1451 DEATAAIDLE TDNLIQATIR TQFDTCTVLT IAHRLNTIMD YTRVLVLDKG

1501 VVAEFDSPAN LIAARGIFYG MARDAGLA*

mrp4.seq Length: 486 September 26, 1996 09:58 Type: N Check: 879 ..

1 acttaaagaa accattgaag atcttcctgg taaaatggat actgaattag

51 cagaatcagg atccaatttt agtgttggac aaagacaact ggtgtgcctt

101 gccagggcaa ttctcaggaa aaatcagata ttgattattg atgaagcgac

151 ggcaaatgtg gatccaagaa ctgatgagtt aatacaaaaa aaaatccggg

201 agaaatttgc ccactgcacc gtgctaacca ttgcacacag attgaacacc

251 attattgaca gcgacaagat aatggtttta gattcaggaa gactgaaaga

301 atatgatgag ccgtatgttt tgctgcaaaa taaagagagc ctattttaca

351 agatggtgca acaacttggc aaggcagaag nnnnnnnnct cactgaaaca

401 gcaaaacngg tatacttcaa aagaaattnt ncacatattg gtgacnctgn

451 ccacatggtt acaaacngtt ncaatggncn nnnntc

FIG. 18a

mrp5a.con Length: 1761 September 10, 1996 11:56 Type: N
Check: 5118 ..

1 CATTGCAATC AGTGGAACCT TCGCTTATGT GGCCCAGCAG
GCTGGATCCT

51 CAATGCTACT CTGAGAGACA ACATCCTGTT TGGGAAGGAA
TATGATGAAG

101 AAAGATACAA CTCTGTGCTG AACAGCTGCT GCCTGAGGCC
TGACCTGGCC

151 ATTCTTCCCA GCAGCGACCT GACGGAGATT GGAGAGCGAG
GAGCCAACCT

201 GAGCGGTGGG CAGCGCCAGA GGATCAGCCT TGCCCGGGCC
TTGTATAGTG

251 ACAGGAGCAT CTACATCCTG GACGACCCCC TCAGTGCCTT
AGATGCCCAT

301 GTGGGCAACC ACATCTTCAA TAGTGCTATC CGGAAACATC
TCAAGTCCAA

351 GACAGTTCTG TTTnnnGTTA CCCACCAGTT ACAGTACCTG
GTTGACTGTG

401 ATGAAGTGAT CTTCATGAAA GAGGGCTGTA TTACGGAAAG
AGGCACCCAT

451 GAGGAACTGA TGAATTTAAA TGGTGACTAT GCTACCATTT
TTAATAACCT

501 GTTGCTGGGA GAGACACCGC CAGTTGAGAT CAATTCAAAA
AAGGAAACCA

551 GTGGTTCACA GAAGAAGTCA CAAGACAAGG GTCCTAAAAC
AGGATCAGTA

601 AAGAAGGAAA AAGCAGTAAA GCCAGAGGAA GGGCAGCTTG
TGCAGCTGGA

651 AGAGAAAGGG CAGGGTTCAG TGCCCTGGTC AGTATATGGT
GTCTACATCC

701 AGGCTGCTGG GGGCCCCTTG GCATTTCCTG GTTATTATGG
CCCTTTTCAT

751 GGCCTGAATG TAGGCAGCAC CGCCTTCAGC ACCTGGTGGT
TGAGTTACTG

801 GATCAAGCAA GGAAGCGGGA ACACCACTGT GACTCGAGGG
AACGAGACCT

FIG. 19a

851 CGGTGAGTGA CAGCATGAAG GACAATCCTC ATATGCAGTA CTATGCCAGC

901 ATCTACGCCC TCTCCATGGC AGTCATGCTG ATCCTGAAAG CCATTCGAGG

951 AGTTGTCTTT GTCAAGGGCA CGCTGCGAGC TTCCTCCCGG CTGCATGACG

1001 AGCTTTTCCG AAGGATCCTT CGAAGCCCTA TGAAGTTTTT TGACACGACC

1051 CCCACAGGGA GGATTCTCAA CAGGTTTTCC AAAGACATGG ATGAAGTTGA

1101 CGTGCGGCTG CCGTTCCAGG CCGAGATGTT CATCCAGAAC GTTATCCTGG

1151 TGTTCTTCTG TGTGGGAATG ATCGCAGGAG TCTTCCCGTG GTTCCTTGTG

1201 GCAGTGGGGC CCCTTGTCAT CCTCTTTTCA GTCCTGCACA TTGTCTCCAG

1251 GGTCCTGATT CGGGAGCTGA AGCGTCTGGA CAATATCACG CAGTCACCTT

1301 TCCTCTCCCA CATCACGTCC AGCATACAGG GCCTTGCCAC CATCCACGCC

1351 TACAATAAAG GGCAGGAGTT TCTGCACAGA TACCAGGAGC TGCTGGATGA

1401 CAACCAAGCT CCTTTTTTTT TGTTTACGTG TGCGATGCGG TGGCTGGCTG

1451 TGCGGCTGGA CCTCATCAGC ATCGCCCTCA TCACCACCAC GGGGCTGATG

1501 ATCGTTCTTA TGCACGGGCA GATTCCCCCA GCCTATGCGG GTCTCGCCAT

1551 CTCTTATGCT GTCCAGTTAA CGGGGCTGTT CCAGTTTACG GTCAGACTGG

1601 CATCTGAGAC AGAAGCTCGA TTCACCTCGG TGGAGAGGAT CAATCACTAC

1651 ATTAAGACTC TGTCCTTGGA AGCACCTGCC AGAATTAAGA ACAAGGCTCC

1701 CTCCCCTGAC TGGCCCCAGG AGGGAGAGGA agAaGGTGAa CsTTTtGAGA

1751 AAGGGGCCTT T

FIG. 19a-2

mrp5b.con Length: 2167 November 19, 1996 14:33 Type: N
Check: 6982 ..

1 TTATGACCmc tGrAnTrrCg CCmAGGGGGs AgAkkrACCn
TTcaTkAgAA

51 GGaaCArAaG aTGgGTACsG AgAAaCtTCc ttTtTTaTCc
tAaAagaaAg

101 TAtyCtttCA CGATCAAACC TAAAGAGAAG ATTGGCATTG
TGGGGCGGAC

151 AGGATCAGGG AAGTCCTCGC TGGGGATGGC CCTCTTCCGT
CTGGTGGAGT

201 TATCTGGAGG CTGCATCAAG ATTGATGGAG TGAGAATCAG
TGATATTGGC

251 CTTGCCGACC TCCGAAGCAA ACTCTCTATC ATTCCTCAAG
AGCCGGTGCT

301 GTTCAGTGGC ACTGTCAGAT CAAATTTGGA CCCCTTCAAC
CAGTACACTG

351 AAGACCAGAT TTGGGATGCC CTGGAGAGGA CACACATGAA
AGAATGTATT

401 GCTCAGCTAC CTCTGAAACT TGAATCTGAA GTGATGGAGA
ATGGGGATAA

451 CTTCTCAGTG GGGGAACGGC AGCTCTTGTG CATAGCTAGA
GCCCTGCTCC

501 GCCACTGTAA GATTCTGATT TTAGATGAAG CCACAGCTGC
CATGGACACA

551 GAGACAGACT TATTGATTCA AGAGACCATC CGAGAAGCAT
TTGCAGACTG

601 TACCATGCTG ACCATTGCCC ATCGCCTGCA CACGGTTCTA
GGCTCCGATA

651 GGATTATGGT GCTGGCCCAG GGACAGGTGG TGGAGTTTGA
CACCCCATCG

701 GTCCTTCTGT CCAACGACAG TTCCCGATTC TATGCCATGT
TTGCTGCTGC

751 AGAgAACAAG GTCGCTGTCA AGGGCTGACT CCTCCCTGTT
GACGAAGTCT

801 CTTTTCTTTA GAGCATTGCC ATTCCCTGCC TGGGGCGGGC
CCCTCATCGC

FIG. 19b

851 GTCCTCCTAC CGAAACCTTG CCTTTCTCGA TTTTATCTTT CGCACAGCAG

901 TTCCGGATTG GCTTGTGTGT TTCACTTTTA GGGAGAGTCA TATTTTGATT

951 ATTGTATTTA TTCCATATTC ATGTAAACAA AATTTAGTTT TTGTTCTTAA

1001 TTGCACTCTA AAAGGTTCAG GGAACCGTTA TTATAATTGT ATCAGAGGCC

1051 TATAATGAAG CTTTATACGT GTAGCTATAT CTATATATAA TTCTGTACAT

1101 AGCCTATATT TACAGTGAAA ATGTAAGCTG TTTATTTTAT ATTAAAATAA

1151 GCACTGTGCT AATAACAGTG CATATTCCTT TCTATCATTT TTGTACnGTT

1201 TGCTGTACnA nAAATCTGGT nTTGCTmTTm nACTGTTAGG AAGAATTAnC

1251 ATTTCATTCT TCTCTAgCTG GTGGTTtCAC gGTGgCCAGG TTTTCTGGGT

1301 GTCCAAAGGA AGACGTGTtG GCAATAGTtn GGGCCCTCCG ACAAGCCCCC

1351 TCTGCCGCCT CCCCACAGCC GCTCCAnGGG GTGGCTGGAG AaCGGGTGGG

1401 CGGCTGGAGA CCATGCCAGA GCGCCGTGAG TTCTCAGGGC TCCTGCCTTC

1451 TGTCCTGGTG TCACTTACTG TTTCTGTtCA GGgAGAGCAG CGGGGCGAAG

1501 CCCAGGCCCC TTTTCACTCC CTCCATCAAG AATGGGGATC ACAGAGACAT

1551 TCCTCCGAGC CGGGGAGTTT CTTTCCTGCC TTCTTCTTTT TGCTGTTGTT

1601 TCTAAACAAG AATCAGTCTA TCCACAGAGA GTCCCACTGC CTCAGGTTCC

1651 TATGGCTGGC CACTGCACAG AGCTCTCCAG CTCCAAGACC TGTTGGTTCC

1701 AAGCCCTGGA GCCAACTGCT GCTTTTTGAG GTGGCACTTT TTCATTTGCC

FIG. 19b-2

1751 TATTCCCACA CCTCCACAGT TCAGTGGCAG GGCTCAGGAT TTCGTGGGTC

1801 TGTTTTCCTT TCTCACCGCA GTCGTCGCAC AGTCTCTCTC TCTCTCTCCC

1851 CTCAAAGTCT GCAACTTTAA GCAGCTCTTG CTAATCAGTG TCTCACACTG

1901 GCGTAGAAGT TTTTGTACTG TAAAGAGACC TACCTCAGGT TGCTGGTTGC

1951 TGTGTGGTTT GGTGTGTTCC CGCAAACCCC CTTTGTGCTG TGGGGCTGGT

2001 AGCTCAGGTG GGCGTGGTCA CTGCTGTCAT CAGTTGAATG GTCAGCGTTG

2051 CATGTCGTGA CCAACTAGAC ATTCTGTCGC CTTAGCATGT TTGCTGAACA

2101 CCTTGTGGAA GCAAAAATCT GAAAATGTGA ATAAAATTAT TTTGGATTTT

2151 GTAAAAAAAA AAAAAAA

FIG. 19b-3

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(71) Applicants *(for all designated States except US)*: INTROGENE B.V. [NL/NL]; Lange Kleiweg 151, NL-2288 GJ Rijswijk (NL). ACADEMISCH MEDISCH CENTRUM AMSTERDAM [NL/NL]; Meibergdreef 9, NL-1105 AZ Amsterdam (NL). HET NEDERLANDS KANKER INSTITUUT [NL/NL]; Plesmanlaan 121, NL-1066 CX Amsterdam (NL).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: OUDE ELFERINK, Ronald, Petrus, Johannes [NL/NL]; Dr. Koomansstraat 27, NL-1391 XA Abcoude (NL). PAULUSMA, Coenraad, Cornelis [NL/NL]; Daendelsstraat 25 bis, NL-3531 GB Utrecht (NL). BOSMA, Piter, Jabik [NL/NL]; Gildstraat 124, NL-3572 ES Utrecht (NL). BORST, Piet [NL/NL]; Meentweg 87, NL-1406 KE Bussem (NL). EVERS, Raymond [NL/NL]; Berlaarstraat 57, NL-1066 PJ Amsterdam (NL). KOOL, Marcel [NL/NL]; Van Walbeeckstraat 52-I, NL-1058 CV Amsterdam (NL).

(74) Agent: SMULDERS, Th., A., H., J.; Vereenigde Octrooibureaux, Nieuwe Parklaan 97, NL-2587 BN The Hague (NL).

(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

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(57) Abstract

The present invention provides a novel family of organic anion transporters of which until now only one member was known. The family includes multispecific organic anion transporters related to the canalicular multispecific organic anion transporter. The cDNA encoding the latter is also provided. The rat and human cDNA are exemplified. Uses of nucleic acids based on this gene family and of cells comprising such nucleic acids as well as vectors comprising sequences thereof are also disclosed especially in the area of gene therapy.

INTERNATIONAL SEARCH REPORT

Inter ional Application No
PCT/NL 97/00079

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/12 C07K14/705 C12Q1/68 A61K48/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	MAYER R ET AL: "Expression of the MRP gene-encoded conjugate export pump in liver and its selective absence from the canalicular membrane in transport-deficient mutant hepatocytes." J CELL BIOL, OCT 1995, 131 (1) P137-50, UNITED STATES, XP000608636 see the whole document ---	1-6,20, 21
X	COLE SP ET AL: "Overexpression of a transporter gene in a multidrug-resistant human lung cancer cell line [see comments]" SCIENCE, DEC 4 1992, 258 (5088) P1650-4, UNITED STATES, XP002017513 see the whole document ---	1-6,20, 21

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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1

Date of the actual completion of the international search	Date of mailing of the international search report
10 July 1997	21. 10. 97

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Gurdjian, D

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/NL 97/00079

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ZAMAN GJ ET AL: "The human multidrug resistance-associated protein MRP is a plasma membrane drug-efflux pump." PROC NATL ACAD SCI U S A, SEP 13 1994, 91 (19) P8822-6, UNITED STATES, XP002017514	1-7, 11-17, 20,21
Y	see the whole document	8-10
X	WO 94 10303 A (UNIV KINGSTON) 11 May 1994 see claims 1-80; figures SEQ.1,2	1-22
Y	WO 92 12987 A (US) 6 August 1992 see page 32, line 26 - page 33, line 22	8-10
A	KITAMURA T ET AL: "Defective ATP-dependent bile canalicular transport of organic anions in mutant (TR-) rats with conjugated hyperbilirubinemia." PROC NATL ACAD SCI U S A, MAY 1990, 87 (9) P3557-61, UNITED STATES, XP002017515 cited in the application see the whole document	1-6
P,X	YI J-R ET AL: "Expression cloning of the cDNA for a polypeptide associated with rat hepatic sinusoidal reduced glutathione transport: Characteristics and comparison with the canalicular transporter" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES OF AMERICA, 92 (5). 1995. 1495-1499., XP002017516 see the whole document	1-6,20, 21
P,X	BUCHLER M ET AL: "cDNA cloning of the hepatocyte canalicular isoform of the multidrug resistance protein, cMrp, reveals a novel conjugate export pump deficient in hyperbilirubinemic mutant rats." J BIOL CHEM, JUN 21 1996, 271 (25) P15091-8, UNITED STATES, XP002017517 see the whole document	1-6,20, 21
P,X	PAULUSMA CC ET AL: "Congenital jaundice in rats with a mutation in a multidrug resistance-associated protein gene." SCIENCE, FEB 23 1996, 271 (5252) P1126-8, UNITED STATES, XP002017518 see the whole document	1-6,20, 21

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/NL 97/ 00079

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claim 23 and claims 16-18, partially as far as they concern an in vivo method, are directed to a method of treatment of the human/aninmal body, a search has been carried out based on the alleged effects of the composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see continuation-sheet

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [X] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1-24 all partially (Subject 1.)

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International Application No. PCT/NL 97 00079

FURTHER INFORMATION CONTINUED FROM PCT/ISA/210

Subject 1: Claims 1-24 (partially): the canicular multispecific organic anion transporter cMOAT protein and gene.

Subject 2: Claims 1-24 (partially): the multidrug resistant-associated protein MRP3 protein and gene.

Subject 3: Claims 1-24 (partially): the multidrug resistant-associated protein MRP4 protein and gene.

Subject 4: Claims 1-24 (partially): the multidrug resistant-associated protein MRP5 protein and gene.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Int. ional Application No
PCT/NL 97/00079

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9410303 A	11-05-94	AU 671160 B	15-08-96
		AU 5173693 A	24-05-94
		AU 682140 B	18-09-97
		AU 7177896 A	06-02-97
		EP 0666911 A	16-08-95
		JP 8504323 T	14-05-96
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WO 9212987 A	06-08-92	AU 1227892 A	27-08-92